

1972

# Carbohydases of *Alcaligenes Faecalis* (Atcc 21400).

Ellen Oliver Smith

*Louisiana State University and Agricultural & Mechanical College*

Follow this and additional works at: [https://digitalcommons.lsu.edu/gradschool\\_disstheses](https://digitalcommons.lsu.edu/gradschool_disstheses)

---

## Recommended Citation

Smith, Ellen Oliver, "Carbohydases of *Alcaligenes Faecalis* (Atcc 21400)." (1972). *LSU Historical Dissertations and Theses*. 2245.  
[https://digitalcommons.lsu.edu/gradschool\\_disstheses/2245](https://digitalcommons.lsu.edu/gradschool_disstheses/2245)

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact [gradetd@lsu.edu](mailto:gradetd@lsu.edu).

## INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.
2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.
3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.
4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

### **University Microfilms**

300 North Zeeb Road  
Ann Arbor, Michigan 48106

A Xerox Education Company

72-28,380

SMITH, Ellen Oliver, 1945-  
CARBOHYDRASES OF ALCALIGENES FAECALIS  
(ATCC 21400).

The Louisiana State University and Agricultural  
and Mechanical College, Ph.D., 1972  
Microbiology

**University Microfilms, A XEROX Company , Ann Arbor, Michigan**

**CARBOHYDRASES OF ALCALIGENES FAECALIS (ATCC 21400)**

**A Dissertation**

**Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy**

**in**

**The Department of Microbiology**

**by  
Ellen Oliver Smith  
B.S., Louisiana State University, 1967  
May, 1972**

PLEASE NOTE:

Some pages may have  
indistinct print.

Filmed as received.

University Microfilms, A Xerox Education Company

To J. R.

## ACKNOWLEDGMENT

The author wishes to express her appreciation to Dr. V. R. Srinivasan for his guidance, patience and understanding during the investigation and preparation of the dissertation. The author wishes to thank Dr. A. D. Larson, Dr. J. M. Larkin and the other members of the Department of Microbiology for their assistance and counsel. A special thanks is extended to Dr. A. R. Colmer who gave this author her love and appreciation for the realm of microbiology. The author also wishes to thank Mrs. M. W. Bumm for her technical assistance.

The author also wishes to thank her husband, John Robert, for his support and patience during the completion of this work.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENT.....	ii
TABLE OF CONTENTS.....	iii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
ABSTRACT.....	ix
INTRODUCTION.....	1
REVIEW OF LITERATURE.....	4
I.    The Transport of Carbohydrates in Bacteria.....	4
The permease concept.....	4
The enzymic nature of the transport phenomenon.....	5
Models for the lactose transport system.....	7
II.   B-glucosidase.....	14
Occurrence.....	14
Association with other carbohydrase activities.....	14
Specificity of B-glucosidases.....	17
Concurrent induction.....	19
MATERIALS AND METHODS.....	21
Organism and medium.....	21
Isolation of mutants.....	21
Preparation of osmotically shocked cells.....	22



Preparation of shock protein.....	23
Uptake of radioactive carbohydrates....	23
Transport specificity.....	24
Scintillation fluid.....	24
Heat inactivation of B-glucosidase and B-galactosidase in crude cell extract.....	25
Extraction and purification of enzymatic activity.....	25
Determination of protein.....	26
Assay of carbohydrase activities.....	26
Alteration of cell permeability.....	26
B-glucosidase assay.....	26
B-galactosidase assay.....	27
Polyacrylamide disc electrophoresis....	28
Amino acid analysis.....	28
Chemicals.....	29
Radioisotopes.....	29
RESULTS.....	30
I. The Transport of Carbohydrates.....	30
Rate of carbohydrate uptake.....	30
K <sub>m</sub> values for the uptake of certain carbohydrates.....	30
Carbohydrate transport in cells grown on succinate.....	33

Inhibitors of uptake of lactose and of a-MG in lactose-induced cells.....	33
The effect of osmotic shock on the uptake of carbohydrates.....	33
II. Evidence for the Presence of Two Carbohydrates.....	37
The effect of CTAB on whole cell activity.....	37
Induction of B-glucosidase and B-galactosidase in whole cells grown on different carbon sources.....	41
Analysis of mutant strains.....	41
Heat inactivation of crude cell extract.....	45
The release of carbohydrase activity from cells subjected to osmotic shock.....	47
The effect of sonication on osmotically shocked cells.....	47
Elutions profiles on gel filtration columns.....	47
III. Purification and Composition of B-glucosidase.....	54
Purification of B-glucosidase.....	54
Preparative polyacrylamide gel electrophoresis.....	54
Amino acid analysis of B-glucosidase.....	54
DISCUSSION.....	58
LITERATURE CITED.....	66
VITA.....	76

## LIST OF TABLES

Table	Page
1. Initial rates of carbohydrate uptake in <u>Alcaligenes</u> cells induced by lactose.....	31
2. $K_m$ values for the uptake of certain carbohydrates by whole cells induced by lactose.....	32
3. Effect of CTAB on whole cell enzymatic activity in lactose-grown cells.....	40
4. Levels of B-glucosidase and B-galactosidase in whole cells grown on different carbon sources.....	42
5. Levels of B-glucosidase and B-galactosidase in wild-type and mutant strains of <u>Alcaligenes</u> grown on cellobiose and on lactose.....	43
6. Release of carbohydrase activity from lactose-induced <u>Alcaligenes</u> during osmotic shock.....	48
7. Purification of B-glucosidase.1.....	55
8. Amino acid composition of <u>A. faecalis</u> B-glucosidase.....	57

## LIST OF FIGURES

Figure	Page
1. Negatively stained preparation of <u>Alcaligenes faecalis</u> (ATCC 21400).....	2
2. Model of the permease system according to Kepes.....	9
3. Model of the B-lac transport system proposed by Koch.....	10
4. Working model of function of M protein in the lac permease system.....	12
5. Uptake of $^{14}\text{C}$ -a-MG and $^{14}\text{C}$ -lactose into uninduced <u>A. faecalis</u> cells.....	34
6. Inhibition of $^{14}\text{C}$ -a-MG uptake in lactose-induced cells.....	35
7. Inhibition of $^{14}\text{C}$ -lactose uptake in lactose-induced cells.....	36
8. The effect of osmotic shock on the active uptake of $^{14}\text{C}$ -lactose into lactose-induced cells.....	38
9. The effect of osmotic shock on the active uptake of $^{14}\text{C}$ -a-MG into lactose-induced cells.....	39
10. Heat inactivation of B-glucosidase and B-galactosidase activities in a whole cell extract of lactose-induced cells.....	46
11. The release of enzymatic activity and of protein from osmotically shocked cells during sonication.....	49
12. Elution profile on gel filtration of concentrated extract of osmotically shocked cells induced by lactose.....	51
13. Elution profile on gel filtration of concentrated extract of osmotically shocked cells induced by salicin.....	52

14.	Elution profile on gel filtration of concentrated extract of osmotically shocked lactose-induced cells after high speed centrifugation.....	53
15.	Polyacrylamide gel electrophoresis of purified B-glucosidase.....	56

## ABSTRACT

The mechanism of carbohydrate utilization in Alcaligenes faecalis (ATCC 21400) was examined. Transport studies in whole cells showed that a number of carbohydrates are actively transported into the organism. Transport of methyl- $\alpha$ -D-glucoside ( $\alpha$ -MG) was constitutive, while the systems for lactose and for B-glucoside transport were inducible. Lysozyme treatment, followed by cold osmotic shock, destroyed the ability of the cells to actively transport both lactose and  $\alpha$ -MG. Lactose transport was competitively inhibited by cellobiose and by salicin. Glucose and cellobiose inhibited the transport of  $\alpha$ -MG.

B-glucosidase and B-galactosidase activities were induced simultaneously in whole cells of A. faecalis either by lactose or by cellobiose. Studies were undertaken to ascertain whether one protein is responsible for both carbohydrase activities or whether each activity resides in a separate protein. Induction studies showed that salicin, a gratuitous inducer, preferentially induced B-glucosidase activity. Nitrosoguanidine-induced mutants which did not utilize lactose were isolated; these mutant strains possessed B-glucosidase to B-galactosidase ratios which differed significantly from those in the wild-type strain induced by the same carbohydrate. Heat inactivation studies on whole cell extracts showed bimodal kinetics. The B-glucosidase was purified by gel filtration of

protein extracted by gentle sonication of osmotically shocked cells. The elution profile of the gel filtration column showed two enzymatically active peaks with different B-glucosidase and B-galactosidase specific activities. The specific activity of the B-glucosidase peak was 1,950 units per gram of protein. These data demonstrated the presence of two separate carbohydrases in A. faecalis with different specificities toward B-glucosides and B-galactosides.

The B-glucosidase was purified to homogeneity by preparative disc gel electrophoresis. The specific activity of the homogeneous protein was 55,000 units per gram of protein. An amino acid analysis was performed on the purified B-glucosidase.

## INTRODUCTION

Han and Srinivasan (28, 29) isolated and characterized two species of bacteria involved in the microbial degradation of sugarcane bagasse. One isolate was identified as a member of the genus Cellulomonas. This organism produced an extracellular cellulase and readily broke down cellulosic materials such as filter paper and bagasse. The second organism, Alcaligenes faecalis (Fig. 1), utilized, as sole carbon source, cellobiose, the end product of cellulose degradation. When both Cellulomonas and Alcaligenes were grown together in mixed culture on cellulose, the growth of both species was enhanced (26). The symbiosis produced an efficient enzyme system for cellulolytic yeasts and fungi (7, 31, 42, 95, 96).

The cellobiose-utilizing organism produced a B-glucosidase (B-D-glucoside glucohydrolase, EC 3.2.1.21), an enzyme which hydrolyses the B-(1,4)-glucosidic linkage. Alcaligenes B-glucosidase was induced almost equally well by cellobiose and by B-MG\*, a gratuitous inducer. Lactose, a B-galactoside, also induces the enzyme at high levels (27). Although the enzyme shows a marked preference for B-glucosidic linkages, it also possessed a degree of hydrolytic activity on lactose (29) and on ONPG (Srinivasan, personal communication). The molecular weight of Alcaligenes faecalis B-glucosidase was estimated to be 120,000 to 160,000 (29).



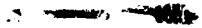


Figure 1. Negatively stained preparation of Alcaligenes  
faecalis (ATCC 21400).



However, the B-glucosidase obtained by Han and Srinivasan (29) was not purified to homogeneity although numerous purification steps were used. Furthermore, since lactose was utilized by the organism as sole carbon source, it was theorized that a second carbohydrase having a greater hydrolytic activity toward B-galactosides than the B-glucosidase may be present in cells which had been grown on lactose. The mechanism of carbohydrate transport in Alcaligenes may also play an intimate part in the coordinate induction phenomenon.

The purposes of this work were to demonstrate two separate carbohydrases in Alcaligenes faecalis (ATCC 21400), to investigate the mechanisms for the transport of carbohydrates in this organism, and to purify B-glucosidase to homogeneity.

---

\*Abbreviations: a-MG, methyl-a-D-glucoside; ATP, adenosine 5'-triphosphate; B-MG, methyl-B-D-glucoside; CTAB, cetyltrimethylammonium bromide; ONPG, o-nitrophenyl-B-D-galactoside; PEP, phosphoenolpyruvate; PNPG, p-nitrophenyl-B-D-glucoside; POPOP, dimethyl-1,4-[2-(4-methyl-5-phenyl-oxazolyl)] benzene; PPO, 2,5-diphenyl-oxazole; TEG, thioethyl-G-D-glucoside; TMG, thiomethyl-B-D-galactoside; TPEG, thiophenylethyl-B-D-galactoside; TPG, thiophenyl-B-D-galactoside.

## REVIEW OF LITERATURE

### I. The Transport of Carbohydrates in Bacteria

The transport of carbohydrates has been studied in a wide variety of organisms and tissues (8, 46, 49, 52, 66, 85). Much of the information concerning the transport phenomenon in bacteria has been accumulated using studies on the B-galactoside transport system of Escherichia coli (49). However, this review will deal with the comparative biochemistry of carbohydrate transport in bacteria only, with special emphasis on the E. coli galactoside transport system.

#### The permease concept

The term "permease" was first applied to the entire transport system by Rickenberg et al. (72). Kepes and Cohen (52) defended this appellation, since it implied a system which was protein in nature and which was similar to an enzyme with regard to specificity and kinetics. At that time, however, no specific protein or its mode of action had been directly demonstrated.

In the 1950's the work of Doudoroff and of Davis, reviewed by Cohen and Monod (9), focused attention on the "crypticity" of certain bacterial systems, i. e., certain cells were unable to utilize a given substrate, although the cell possessed the necessary metabolic enzymes. Often crypticity is highly stereospecific and may involve meta-

bolic intermediates normally found within the cell as breakdown products of utilizable substrates. Cell-free extracts of the so-called cryptic cells, on the other hand, readily broke down or oxidized the compounds to which the whole cells were impermeable.

The galactoside permease system in Escherichia coli was the first permease system studied in depth and much of our present knowledge of the transport phenomenon has been extrapolated from the studies on this system. It was shown that the galactoside permease was specific for all B-galactosides and B-thiogalactosides (52). The permease system is also specific for the conditions of its induction, since only B-galactosides and B-thiogalactosides induce its synthesis (72); the substrate specificity of permease is apparently similar to that of B-galactosidase (9), although the gene responsible for some essential component of this permease system is at a locus distinct from the structural gene for B-galactosidase (49, 52).

#### The enzymic nature of the transport phenomenon

Rickenberg et al. (72) found that the accumulation of TMG, a galactoside which cannot be hydrolyzed by B-galactosidase in E. coli, was dependent on the concentration of the substrate in the surrounding medium and was analogous to the affinity of an enzyme for its substrate. A double reciprocal plot of levels of accumulated TMG versus the external concentration of substrate resulted in

a straight line, as in the Lineweaver-Burk treatment of enzyme saturation kinetics. TPEG and TPG competitively inhibited TMG accumulation.

Rickenberg et al. (72) described the relationship between the external concentration of substrate ( $G_{ex}$ ) and the amount of substrate accumulated within the cell at equilibrium ( $G_{in}$ ) by an equation similar to the Michaelis-Menten equation:

$$G_{in} = Y \frac{G_{ex}}{G_{ex} + K_t}$$

Y is the "capacity" of the cell or the maximal amount of galactoside which the cells take up at saturating concentrations, and  $K_t$  is the concentration of external galactoside which half-saturates the system (49).

Kepes and Monod, as cited Kepes and Cohen (52), showed that the initial rate of uptake of galactoside is also a function of the external substrate concentration:

$$v_{in} = v_{in}^{max} \frac{G_{ex}}{G_{ex} + K_t}$$

Furthermore, in the steady state, the exit of substrate is a linear function of external concentration:

$$v_{ex} = G_{in} \cdot k_{ex}$$

where  $k_{ex}$  is a first order rate constant for the rate process.

The exit process is not the result of non-specific leakage as Rickenberg et al. (72) first hypothesized. Several independent investigators have since shown that the exit of galactosides in E. coli is a carrier-mediated process (51, 54, 93). The rate of exit of galactoside is greatly reduced by p-chloromecuribenzoate, a sulfhydryl reagent which blocks carrier-mediated transport of galactoside into the cell (51). The temperature coefficient of the exit process is high, as is that of the entry process (54). Furthermore, the apparent  $K_t$  for the exit process depends on the energy metabolism of the cell; the exit of lactose and of ONPG from pre-loaded cells was a saturable process when the cells were poisoned with sodium azide plus iodoacetate (93).

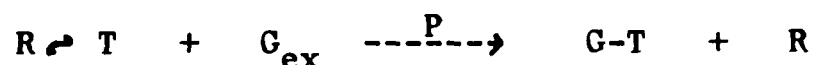
#### Models for the lactose transport system

Two general classes of models have been proposed to account for the transport of galactosides in E. coli cells. The first type of model explains the accumulation process as the result of a cycle of transformation of the sugar itself (51, 54). The carrier, not the substrate, undergoes cyclic interconversions in the second type: transitions of the carrier reduce its affinity for substrate on the inner side of the cytoplasmic membrane (25, 93).

The transporter model proposed by Kepes (51) in 1960

is shown in Fig. 2. The galactoside moiety (G) crosses the membrane barrier only after it has combined with a substance of unspecified chemical nature called the "transporter" (T). The sugar-transporter complex (G-T) then freely crosses the membrane barrier.

According to Kepes' model, the permease protein (P) is responsible for the catalysis of a reaction between an activated form of the transporter (R-T) and the sugar ( $G_{ex}$ ):



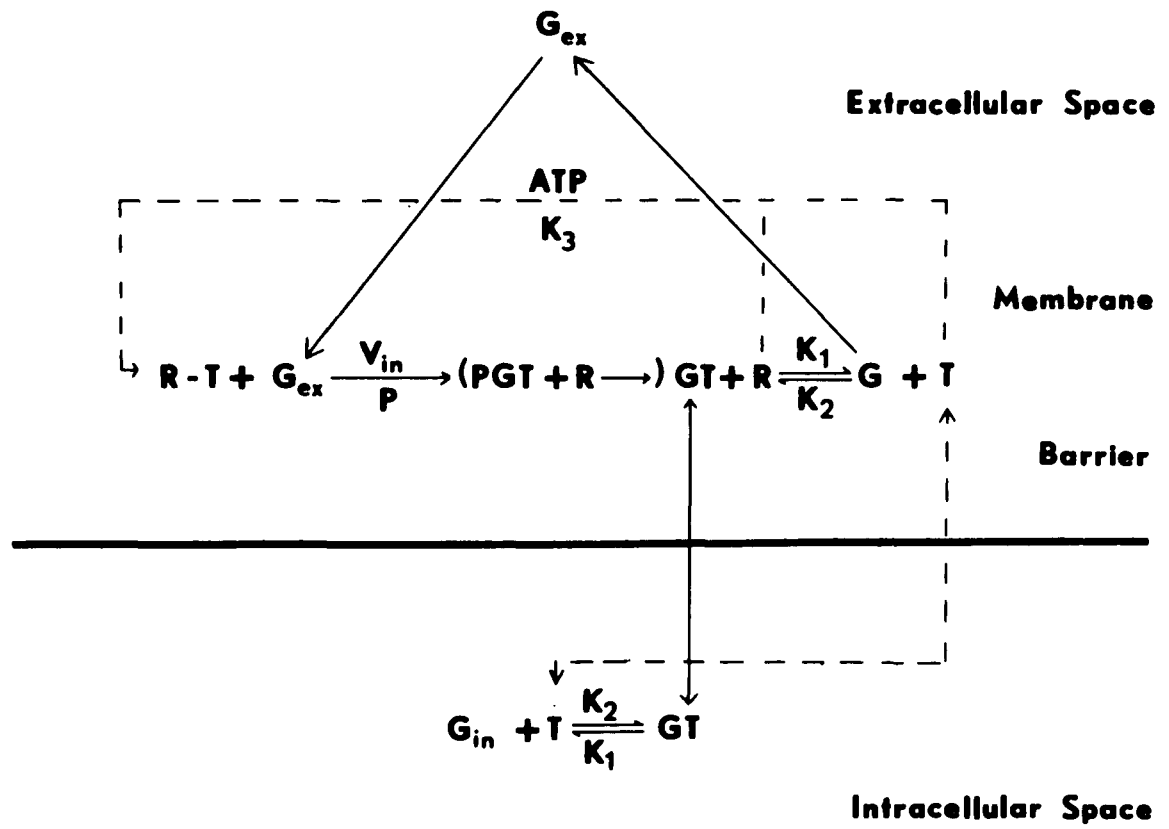
This process is irreversible. This reaction is also postulated to be the rate-limiting step for the entire process. G-T then traverses the membrane barrier freely and may dissociate on either side of the membrane. This reversible association-dissociation, combined with the diffusion step, provides an explanation for the carrier-mediated exit of the sugar. Activated transporter is regenerated by the utilization of ATP.

Kepes maintains that metabolic energy is a fundamental requirement for the entry of galactosides. Most investigators now disagree with this hypothesis. Accumulation requires metabolic energy, but permease mediated entry is independent of metabolic energy (23, 25, 54, 71).

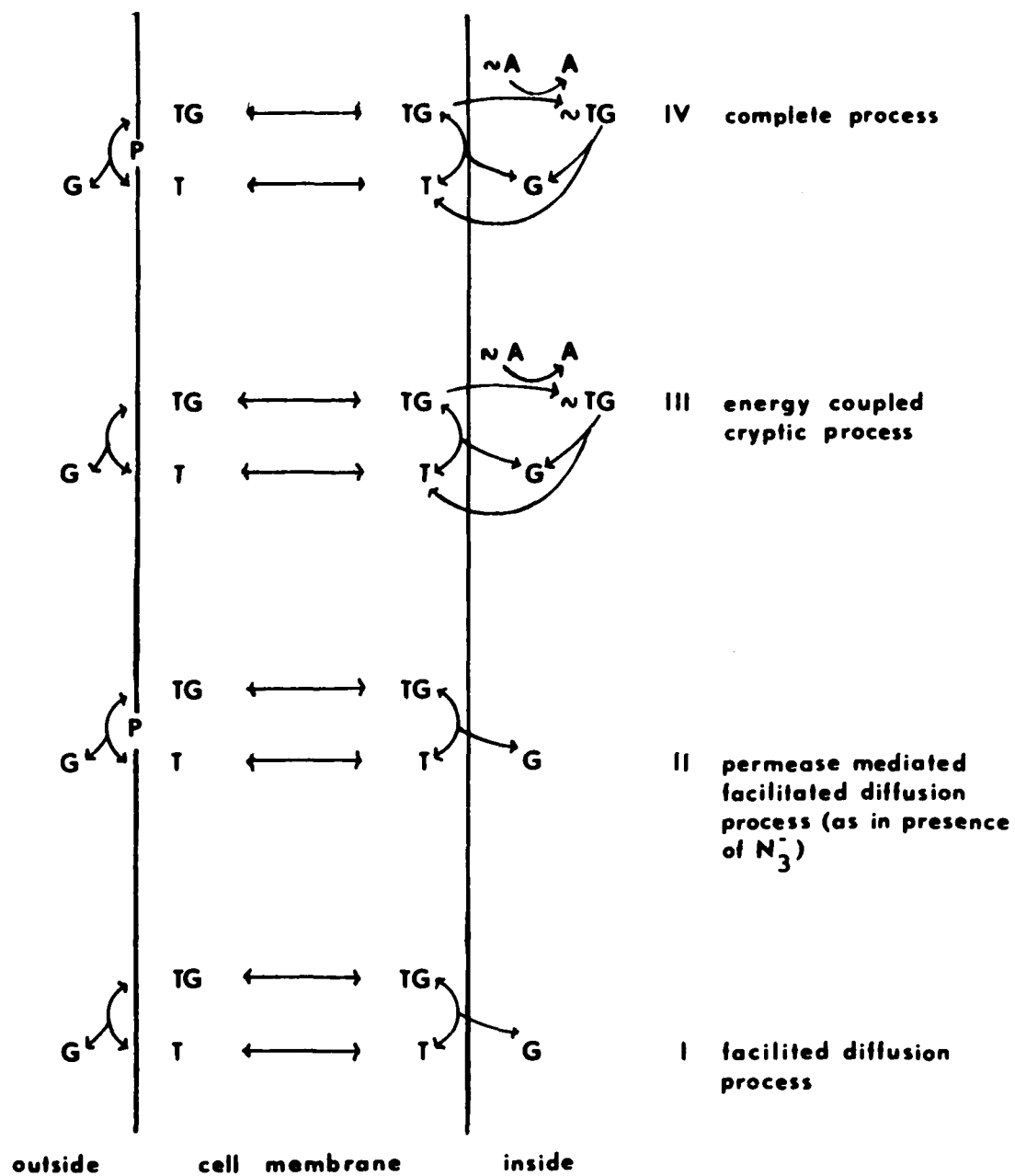
Kepes' model was extended and revised by Koch (54, 55). Fig. 3 illustrates Koch's concept of the transport process, both in permease-containing and cryptic cells. According



**Figure 2. Model of the permease system according to Kepes (57).**



**Figure 3. Model of the B-lac transport system proposed by Koch (54, 55).**

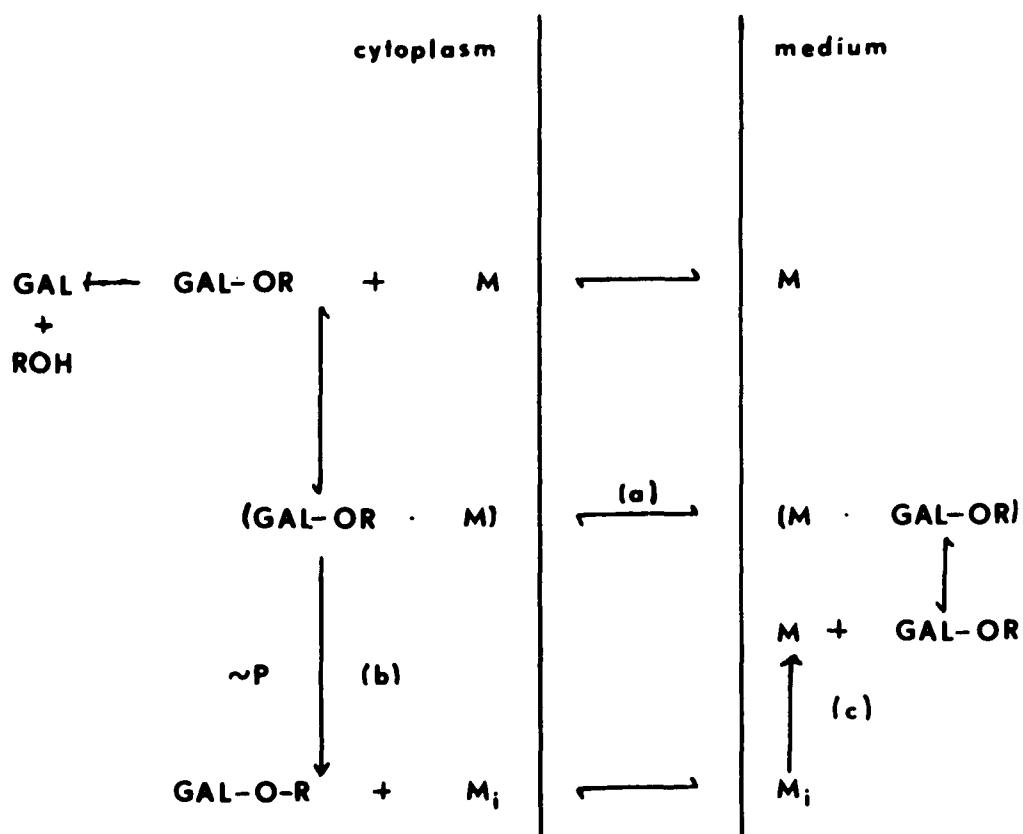


to Koch, "the transporter (T) is the substance, structure, or channel that permits the diffusion of the substrate (G) by its interaction, through the cell membrane." Metabolic energy is released by the conversion of compound A (presumably ATP) to compound B in an exergonic reaction when the substrate is actively extracted from the activated transporter-substrate complex (TG) by a subsystem. The permease (P) catalyzes the attachment of the substrate to the transporter. Hence, as in condition II, Fig. 3, the facilitated entry of the galactoside into the cell does not require energy, since this process may occur even in energy-poisoned cells, although the cell contains permease. The "complete" process of galactoside accumulation (condition IV, Fig. 3), on the other hand, does require metabolic energy for the function of the subsystem. The exit process is a reversal of the uncatalyzed dissociation of the transporter-substrate complex at the inside of the cell membrane (condition I and condition II).

Fox and Kennedy (25) in 1965 devised a model which is based on the hypothesis that the transporter, not the substrate undergoes a series of cyclic transformations. The working model (Fig. 4) distinguishes between two aspects of the transport phenomenon: (a) the facilitated entry of the substrate into the cell, and (b) the accumulation of galactoside against a concentration gradient.

A galactoside molecule combines with the M protein

Figure 4. Working model of function of M protein in the  
lac permease system. From Fox and Kennedy  
(25).



at the exterior surface of the membrane. It then passes through the membrane as a B-galactoside-M complex [reaction (a)]. Once inside the membrane barrier, the complex may simply dissociate. If B-galactosidase is present and if the B-galactoside serves as a substrate for the enzyme, the hydrolysis of the substrate continuously removes the galactoside, resulting in a substantial uptake of galactoside. This process does not require metabolic energy, since it may be demonstrated in energy-poisoned cells (54). However, if sources of metabolic energy are available to the cell, component M is converted, via reaction (b), to  $M_i$ , a form with a reduced affinity for B-galactosides.  $M_i$  moves back through the membrane to the outside surface, where it is converted to M through a reaction which does not require the expenditure of metabolic energy [process (c)]. The maintenance of a concentration gradient, *i. e.*, internal accumulation of B-galactoside, requires the utilization of energy (25).

This model does not involve the generalized transporter or sugar carrier substance as do those proposed by Kepes (51) and Koch (54, 55). Instead, the sugar binds directly to the M protein. Although B-galactoside-M protein binding data (49) support the M protein model of Fox and Kennedy (25), the necessary cycle of M protein transformations has not been demonstrated in cell-free systems. Indeed a conformational change in the M protein would be



difficult to demonstrate in vitro should this be the only transformation in that protein.

## II. B-glucosidase

### Occurrence

Many of the classical attempts at the purification of this glycolytic activity were made with almond "emulsin" (26, 37, 38), an extract of almonds possessing the ability to hydrolyze the B-glucoside amygdalin into glucose, benzaldehyde and hydrocyanic acid (92). B-glucosidases have also been isolated and characterized from animal and plant sources (1, 11, 13, 14, 41, 48, 64), as well as yeasts (17, 42, 47, 65, 96), fungi (7, 18, 19, 30, 31, 40, 53, 83, 84, 89, 93), and bacteria (16, 27, 29, 76).

### Association with other carbohydrase activities

B-glucosidase activity is often associated with other carbohydrases. The enzymatic breakdown of cellulose, a B-1,4 polymer of glucose, is carried out by a variety of cellulolytic organisms. Usually one or more components of these cellulolytic systems are B-glucosidases which hydrolyze oligosaccharide breakdown products resulting from enzymatic degradation of the cellulose molecule (7, 19, 42, 53, 56, 83, 84, 88, 89, 95). Neurospora crassa synthesizes two B-glucosidases and two cellulases which are separable by ammonium sulfate fractionation (19). Cellulase and B-glucosidase components in culture filtrates of the

wood stain fungus Botryodiplodia theobromae can be separated by polyacrylamide disc gel electrophoresis and by gel filtration (90). The B-glucosidase in Poria vaillantii has a markedly different heat stability than does a second component of the Poria cellulose-utilizing system (83); the B-glucosidase from Myrothecium verrucaria exhibits properties similar to the Poria enzyme (56). Stachybotrys atra produces two B-glucosidases distinct from cellulase (96). The cellulolytic enzyme system of Trichoderma koningii also has a B-glucosidase separate from cellulase (95).

In other systems of B-glucosidase may occur in conjunction with such carbohydrases as B-galactosidase,  $\alpha$ -galactosidase, B-N-acetylglucosaminidase, and B-D-fructosidase. B-glucosidase in barley and in the limpet, Patella vulgata, are distinct from B-galactosidase, as is shown by lactone-inhibition studies (12). Cell extracts of Schizophyllum commune produce three B-glucosidases, each with unique specificities (93). A number of glycosidases have been isolated from Phaseolus vulgaris; B-glucosidase activity was found to be separate from B-galactosidase (3). Cultured sycamore cells possess a number of separate carbohydrases:  $\alpha$ -galactosidase,  $\alpha$ -mannosidase, B-N-acetyl-glucosaminidase, B-glucosidase, and two B-galactosidases (48). B-glucosidase is also among those activities characterized in a group of bacteri-

olytic enzymes from Acanthamoeba castellanii (74). Almond emulsin has been shown to contain at least two enzymes with B-glucosidic activity (26).

B-galactosidase cannot always be separated from B-glucosidase preparations, however. Heyworth and Walker (37, 38) showed that one protein possessed both activities in sweet-almond emulsin. Emulsin B-glucosidase and B-galactosidase activities had approximately the same pH optima and could not be separated by ammonium sulfate fractionation (37). Furthermore, several inhibitors were competitive inhibitors with similar affinities against enzyme substrates (38). Conchie et al. (12) supported the identity of B-glucosidase and B-galactosidase in almond emulsin and demonstrated the presence of a third enzymatic activity B-D-fucosidase at the same catalytic site through lactone-inhibition studies. Human kidney B-glucosidase also possesses B-galactosidase activity (14). B-glucosidase and B-galactosidase activities from pig intestine formed a single homogeneous peak when eluted from a triethylaminoethyl (TEAE) cellulose column; the ratio of activities in the peak was the same as that in crude homogenate (13). Pig kidney contains a B-glucosidase which is active toward a number of carbohydrates including B-galactosides: these activities cannot be separated by gel electrophoresis, column chromatography or gel filtration (1). B-galactosidase activity cannot be removed totally from preparations

of Sorghum B-glucosidases, indicating some overlapping specificity (64).

### Specificity of B-glucosidases

Aizawa (4) first used the aryl B-glucoside p-nitro-phenyl-B-D-glucoside (PNPG) as a substrate for the assay of B-glucosidase activity. The release of the chromogenic moiety p-nitrophenol from the parent compound by B-glucosidase is readily followed spectrophotometrically in the visible range. PNPG is also readily hydrolyzed by all B-glucosidases (26, 83, 90), probably due to the electronegativity of the nitro group which decreases the stability of the B-glucosidic linkage. A number of B-glucosidases, however, will attack only the glucosidic bond in PNPG and other aryl-B-glucosides but do not hydrolyze cellobiose or aliphatic B-glucosides. Systems containing more than one B-glucosidase activity often have at least one enzyme exhibiting this type of specificity.

Jermyn (42) studied a strain of Stachybotrys atra which secreted B-glucosidase into the medium. Some preparations with strong cellobiase activity also showed a higher salicinase to PNPGase ratio than those preparations without cellobiase. Youatt (96) confirmed the presence of two distinct B-glucosidases produced by S. atra in addition to cellulase: (1) an aryl B-glucosidase, and (2) a cellobiase. The intracellular cellobiase hydrolyzed PNPG but had little activity toward methyl B-glucoside. It readily

attacked oligosaccharides from cellobiose to cellopentose and a cellodextrin ( mean chain length 11) (96). Laminarinase was also associated with cellobiase activity (42).

Jermyn purified and characterized the S. atra aryl B-glucosidase (42, 43, 44, 45). TPG served as a substrate for the enzyme. It had a higher affinity for aryl B-glucosides than for alkyl B-glucosides and did not attack cellobiose (43). The enzyme had no activity towards  $\alpha$ -D-glucosides,  $\alpha$ - and B-D-galactosides,  $\alpha$ - and B-D-mannosides, B-D-xylosides, B-L-arabinosides, or cellulose (45).

Neurospora crassa also possesses two B-glucosidases (18, 63). Like the system in Stachybotrys, cellobiase activity is internal and cryptic. Intact mycelia cannot utilize cellobiose (17). The cellobiase is also heat labile, loosing all of its activity within two minutes at 60 C (63). The thermostable aryl B-glucosidase (63) activity in N. crassa is almost equally divided between extracellular and bound activities (17).

B-glucosidases from culture filtrates of Myrothecium verrucaria include at least three enzymes which hydrolyze PNPG (30, 31). One of the components isolated by paper electrophoresis was truly an aryl B-glucosidase, since it was inactive on cellobiose. A second activity corresponded with cellobiase activity. The extracellular enzyme system of another fungus, Stereum sanguinolentum, also shows similar distribution of B-glucosidic activities: (1) an

aryl B-glucosidase without cellobiase activity, (2) a cellobiase which appears not to be an aryl B-glucosidase, and (3) an enzyme with both activities that cannot be separated (7).

#### Concurrent induction

A number of carbohydrases may be induced by compounds which do not serve as substrates. Monod et al. (67) reported that two  $\alpha$ -D-galactosides, methyl- $\alpha$ -D-galactoside and melibiose (6-glucose- $\alpha$ -D-galactoside) serve as gratuitous inducers of Escherichia coli B-galactosidase. These results were incompatible with all of the hypotheses which had been proposed up to that time and which had implied that induction was connected with enzymatic activity or with the formation of a specific complex between the enzyme and the inducer. Landman (59) demonstrated that the B-galactosidase of Bacillus megaterium was also induced by melibiose.

Shenin and Crocker (80) investigated the concurrent induction of  $\alpha$ -galactosidase and B-galactosidase in E. coli B. Inducers of galactosidase activity fell into three groups: (1) galactose, aliphatic  $\alpha$ - and B-galactosides, melibiose and raffinose concurrently induce the formation of both  $\alpha$ - and B-galactosidase; (2) aryl- $\alpha$ -galactosides, o-nitrophenyl- $\alpha$ -D-galactoside and  $\alpha$ -phenyl galactoside induce the formation of only  $\alpha$ -galactosidase; (3) only B-galactosidase is induced by B-galactosides, lactose,

ONPG and TMG. Furthermore, although several galactosides induced both a- and B-galactosidases, the relative rate of synthesis of each activity was different for different inducers.

The induction of a- and B-glucosidases of Klebsiella aerogenes follows a pattern similar to the induction pattern of a- and B-galactosidases in E. coli (16). Maltose induces the greatest levels of a-glucosidase, but about 40 per cent of this level was observed in organisms from a cellobiose-limited system. Cells grown in cellobiose-limited conditions had high B-glucosidase activities. However, melibiose-grown cells had about 50 per cent of this activity.

B-glucosidase may also be induced by B-galactosides. Lactose serves as an inducer for extracellular and intracellular PNPGase activities in mushrooms (93). Salmonella variants which ferment lactose also ferment cellobiose; these variants generally ferment arbutin and salicin. Conversely, those cells initially cultured on cellobiose ferment lactose (78, 79).

## MATERIALS AND METHODS

### Organism and medium

All experiments were performed with a strain of Alcaligenes faecalis (ATCC 21400). The organism was grown in a basal salts medium which contained (per liter distilled water): NaCl, 3.0 g;  $(\text{NH}_4)_2\text{SO}_4$ , 1.0 g;  $\text{KH}_2\text{PO}_4$ , 0.5 g;  $\text{K}_2\text{HPO}_4$ , 0.5 g;  $\text{MgSO}_4$ , 0.1 g; and  $\text{CaCl}_2$ , 0.1 g. Carbon sources and inducers were added as indicated. Cells were grown at 30 C on a gyrorotary shaker and harvested in the late exponential growth phase unless otherwise specified.

Stock cultures were maintained on basal salts agar slants with 0.1% cellobiose.

Batch cultures for the purification of B-glucosidase were grown in 20 l carboys of basal salts medium containing 2% sodium succinate, 0.5% lactose and 0.1% yeast extract. Cultures were grown under forced aeration at 30 C and harvested in a steam-driven, cooled Sharples Super-Centrifuge at 40,000 rpm.

### Isolation of mutants

An exponentially-growing culture in basal salts medium plus 0.1% lactose was diluted 1:2 into fresh medium supplemented with 0.1% yeast extract, 0.03% beef extract, and 0.05% peptone. The culture was incubated at 30 C for one hour, and then 500 ug per ml of N-methyl-N'-nitro-N-nitrosoguanidine was added. The culture was incubated for three hours at 30 C and was then centrifuged and washed



once with chilled nutrient broth. The cells were suspended in fresh basal salts medium and plated on basal salts agar supplemented with 0.5% casamino acids. The plates were incubated at 30 C for 48 hours. Replicas of the casamino acids-basal salts agar plates were then made onto basal salts agar containing 0.1% lactose, according to the method of Lederberg and Lederberg (61). The lactose mutants selected were those which failed to grow on the lactose-basal salts medium or those which grew so slowly that they did not form visible colonies as rapidly as did the wild-type strain.

#### Preparation of osmotically shocked cells

Cells were subjected to modified osmotic shock according to the procedure of Nossal and Heppel (69) and Neu and Heppel (68). A one-liter culture of cells was harvested, and the cells were washed twice with 0.01 M Tris-HCL buffer, pH 7.4. The cells were then suspended in 40 ml of the same buffer and 40 ml of 40% sucrose in 0.01 M Tris-HCL buffer, pH 7.4, was added. Crystalline lysozyme was dissolved in the cell suspension to a final concentration of 1 mg per ml. The suspension was incubated, with stirring, at 37 C for 10 min. Following centrifugation in the cold, the cells were rapidly suspended in  $5 \times 10^{-4}$  M  $MgCl_2$  and incubated at 4 C for 20 min. The osmotically shocked cells were then removed from the dilute  $MgCl_2$  solution by centrifugation in the cold. The supernatant fluid from this centrifugation will be

referred to as shock supernatant fluid.

#### Preparation of shock protein

Protein from the shock supernatant fluid was concentrated by precipitation with ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation at 40,000 x g in a Sorvall RC-2 refrigerated centrifuge. The precipitate was dissolved in 1.0 ml of 0.1 M sodium phosphate buffer, pH 6.5, and dialyzed overnight against the same buffer. The concentrated, dialyzed protein shall be designated shock protein.

#### Uptake of radioactive carbohydrates

Uptake of radioactive carbohydrates by A. faecalis was performed according to a modification of the method of Egan and Morse (20). One-liter overnight cultures were grown on basal salts medium containing 0.5% sodium succinate and 0.1% lactose. Cells were harvested and suspended in 100 ml of fresh growth medium. Following incubation at 30 C for 1.5 h, the cell suspension was centrifuged and washed once with basal medium. The cells were resuspended in 100 ml basal salts medium containing 0.5% succinate. Chloramphenicol was added (25 ug/ml), and the cell suspension was incubated at 30 C for 30 min to inhibit protein synthesis. Radioactive substrate was added to the suspension, and samples (1.0 ml) were taken at increasing time, filtered through selectron membrane filters, type B-6 (Schleicher and

Schuell Inc.), and washed with cold basal medium. The filters were dried, and the samples were counted in a Beckman Liquid Scintillation spectrometer.

In studies which contrasted uptake in osmotically shocked cells and in intact cells, cells from a single culture were divided into two aliquots, one of which was subjected to osmotic shock. Uptake assays were then performed on both aliquots as described above.

#### Transport specificity

The specificity of carbohydrate transport in the organism was studied by the measurement of the affinity of a number of carbohydrates for both the lactose and the a-MG transport systems. Inhibition of transport was determined at a fixed concentration of inhibitor ( $1 \times 10^{-3}$  M) over a wide range of lactose and of a-MG concentrations. Initial velocities of carbohydrate uptake were plotted against substrate concentration in a double reciprocal plot.

#### Scintillation fluid

Scintillation fluid for radioactive experiments was prepared according to the following formula: naphthalene, 60 g; PPO, 4 g; POPOP, 200 mg; absolute methanol, 100 ml; p-dioxane, to 1 l. Ten ml of this fluid was used in each scintillation vial.

### Heat inactivation of B-glucosidase and B-galactosidase in crude cell extract

Nine ml of 0.1 M sodium phosphate buffer, pH 6.5, was equilibrated at 55 C in a water bath. One ml of crude cell extract was added. Samples (0.5 ml) were removed at various time intervals and blown into prechilled test tubes. Samples were assayed for enzymatic activity after they had been equilibrated at 40 C.

### Extraction and purification of enzymatic activity

Osmotically shocked cells were suspended in a small volume of cold 0.01 M sodium phosphate buffer, pH 6.5. An extract was prepared by treatment in a Branson sonic disintegrator for 60 sec, followed by low speed centrifugation to remove the cell debris and intact cells. Protein in the supernatant fluid was concentrated by precipitation of the protein with ammonium sulfate at 70% saturation. The precipitated protein was suspended in 0.1 M sodium phosphate buffer, pH 6.5, and dialyzed overnight against the same buffer.

Five ml samples of concentrated extract were then loaded on gel filtration columns (Sephadex G-100) to separate enzymatic activities. Columns (2.0 x 100 cm) were prepared from deaerated gel which had been washed and swelled in a boiling water bath according to the manufacturer's recommendations. The protein was eluted with 0.1 M sodium phosphate buffer, pH 6.5, containing  $1 \times 10^{-4}$  M  $\text{MnCl}_2$ , which was

added to help stabilize enzyme activity. One-hundred drop fractions were collected and assayed for protein, B-glucosidase activity and B-galactosidase activity.

#### Determination of protein

Quantitative protein determinations were made according to the method of Lowery et al. (62), using Folin-Ciocalteu phenol reagent. Crystalline bovine serum albumin was used as the standard. Protein estimations during the purification process were made by the ratio of absorbance at 280 nm to that at 260 nm, based on the nomograph prepared by Adams (2).

#### Assay of carbohydrase activities

Alteration of cell permeability. Ulitzur (87) found that CTAB, a surfactant, was as effective in the alteration of cell permeability as other previously described methods. One-tenth ml of CTAB solution ( 2 mg/ml) was added to 0.4 ml of whole cells which had been suitably diluted in 0.1 M sodium phosphate buffer, pH 6.5, and the cell suspension was incubated for 10 min at 40 C before enzyme assays were made. All whole cell assays were carried out in the presence of CTAB unless otherwise specified.

B-glucosidase assay. B-glucosidase activity was estimated by a modification of the method of Aizawa (4). The substrate PNPG is hydrolyzed by B-glucosidase to give glucose and the chromogenic compound p-nitrophenol, which can be

followed spectrophotometrically. Five-tenths ml of enzyme solution, suitably diluted in 0.1 M sodium phosphate buffer, pH 6.5, or of CTAB-treated whole cells was mixed with 0.5 ml of  $2 \times 10^{-3}$  M PNPG in 0.1 M sodium phosphate buffer, pH 6.5. The mixture was incubated at 40 C until the appearance of yellow color. The reaction was stopped by the addition of 2.0 ml of 0.25 M sodium carbonate. The reaction mixture was centrifuged if it contained whole cells, and the supernatant was decanted. The absorbance of the supernatant solution or of the entire reaction mixture was then read at 400 nm on a Beckman DB spectrophotometer. Units of B-glucosidase are expressed as nmoles PNPG hydrolyzed per minute under the specified conditions. Specific activity is units of B-glucosidase per gram of protein.

B-galactosidase assay. A modification of the method of Lederberg (60) and Rickenberg et al. (73) was used for B-galactosidase assay. B-galactosidase activity was followed by the release of o-nitrophenol from ONPG. Five-tenths ml of enzyme dilution or cell suspension, previously treated with CTAB, was incubated with 0.1 ml of 12 mM  $\text{MnCl}_2$  and 0.4 ml of  $2 \times 10^{-3}$  M ONPG in 0.1 M sodium phosphate buffer, pH 6.5, at 40 C until the appearance of yellow color. The reaction was then treated as described above for the B-glucosidase assay. Units of B-galactosidase are expressed as nmoles ONPG hydrolyzed per minute under the above conditions; specific activity is equal to units B-galactosidase per gram

protein.

### Polyacrylamide disc electrophoresis

Polyacrylamide disc electrophoresis was performed as described by Davis (15) and Ornstein (70). Samples were placed in sucrose rather than in a large pore sample gel. Bromphenol blue was utilized as the standard tracking dye.

Analytical gels were prepared in 5 x 115 mm tubes. A current of 5 ma per gel was supplied by a Heathkit Regulated Power Supply, Model 1P-32. Completed gels were then routinely stained for protein with 1% amido black 10B (buffalo black) in 7% acetic acid. Gels were destained in 7% acetic acid on a reciprocating shaker.

Preparative gel electrophoresis of concentrated extract from osmotically shocked cells was performed on a Buchler "Poly-Prep" preparative polyacrylamide gel electrophoresis apparatus (Buchler Instrument Inc., Fort Lee, New Jersey) according to the manufacturer's instructions. The resolving gel measured 6 cm in length. A Beckman/Spinco Constant Regulated Power Supply operating at 50 ma (constant current) was used.

### Amino acid analysis

An amino acid analysis of A. faecalis B-glucosidase was carried out on a Beckman/Spinco Model 120 C amino acid analyzer. Protein samples were lyophilized and prepared for analysis by hydrolyzing with HCl under vacuum

for 24, 48 and 72 hours at 105 C. After hydrolysis the samples were dried over concentrated  $\text{H}_2\text{SO}_4$  and NaOH pellets. The samples were then dissolved in 0.2 M citrated buffer, pH 2.2 and filtered with a Swinny filter immediately prior to analysis.

### Chemicals

N-methyl-N'-nitro-N-nitrosoguanidine was purchased from Aldrich Chemical Co. a-MG, PNPG and ONPG were products of Calbiochem. CTAB, p-dioxane, N,N'-methylenebisacrylamide, acrylamide, N,N,N',N'-tetramethylethylenediamine and riboflavin were purchased from Eastman Organic Chemicals. Naphthalene was purchased from Matheson Coleman and Bell. Packard Instrument Co. supplied PPO and POPOP. a-MG, Tris(hydroxymethyl)aminomethane (Sigma 7-9), and crystalline lysozyme were products of Sigma Chemical Co.

### Radioisotopes

L-leucine- $^{14}\text{C}$  (U) was supplied by New England Nuclear Corp. Amersham-Searle supplied the following labelled carbohydrates: maltose (U), methyl [ $\alpha$ -D-glucose- $^{14}\text{C}$  (U)] pyranoside, D-mannitol-1- $^{14}\text{C}$ , lactose-1- $^{14}\text{C}$ , sucrose- $^{14}\text{C}$  (U), D-galactose-1- $^{14}\text{C}$ , D-glucose-1- $^{14}\text{C}$ , and D-fructose- $^{14}\text{C}$  (U).



## RESULTS

### I. The Transport of Carbohydrates

#### Rate of carbohydrate uptake

The initial rate of uptake for a number of carbohydrates is summarized in Table 1. Mannitol uptake followed a straight line, while the remainder of the carbohydrates reached levels of saturation within 90 minutes. Egan and Morse (20) reported similar comparative studies of carbohydrate uptake in S. aureus in which mannitol alone gave a straight line graph for uptake versus time. S. aureus cells induced with lactose also showed similar levels of galactose uptake.

#### K<sub>m</sub> values for the uptake of certain carbohydrates

The uptake of carbohydrates by whole cells was found to be a saturable process (Table 2). The respective Michaelis constants for saturation were calculated from Lineweaver-Burk plots of velocity and external carbohydrate concentration. The K<sub>m</sub> values for α-MG and for glucose are of the same order of magnitude and the same absolute value. Egan and Morse (22) found Michaelis constants for K<sub>m</sub> = 5 x 10<sup>-6</sup>M for lactose; K<sub>m</sub> = 2 x 10<sup>-5</sup>M for α-MG; and for maltose K<sub>m</sub> = 1 x 10<sup>-4</sup>M in S. aureus. S. aureus has a lower K<sub>m</sub> for the transport of lactose than does A. faecalis, but the values for α-MG and maltose transport are similar.

Table 1

Initial rates of carbohydrate uptake in  
Alcaligenes cells induced by lactose

Carbohydrate	Initial concentration of carbohydrate	Initial rate of uptake (umoles/g dry wt per 10 minutes)
a-MG	$1.5 \times 10^{-5}$ M	3.0
Lactose	$1.2 \times 10^{-5}$ M	3.7
Maltose	$2.3 \times 10^{-5}$ M	52.6
Sucrose	$5.6 \times 10^{-6}$ M	6.7
Fructose	$2.9 \times 10^{-5}$ M	80.3
Mannitol	$5.4 \times 10^{-5}$ M	14.9
Glucose	$7.3 \times 10^{-6}$ M	5.7
Galactose	$1.4 \times 10^{-5}$ M	1.1

<sup>a</sup> Initial rate of uptake is linear over first 10 minutes.

Table 2

$K_m$  values for the uptake of certain carbohydrates  
by whole cells induced by lactose

Carbohydrate <sup>a</sup>	$K_m$ (moles/liter)	$V_{max}$ (moles/10 <sup>min</sup> per g dry wt.)
a-MG	$3.2 \times 10^{-5}$	$2.2 \times 10^{-5}$
Lactose	$8.3 \times 10^{-5}$	$5.3 \times 10^{-6}$
Glucose	$4.9 \times 10^{-5}$	$4.5 \times 10^{-6}$
Galactose	$1.1 \times 10^{-4}$	$4.5 \times 10^{-6}$
Maltose	$2.3 \times 10^{-4}$	$4.8 \times 10^{-5}$
Sucrose	$3.3 \times 10^{-4}$	$8.3 \times 10^{-5}$

### Carbohydrate transport in cells grown on succinate

Cells grown on 2% succinate actively transport a-MG but fail to transport lactose (Fig. 5). The a-MG transport system in Alcaligenes is therefore constitutive, while the lactose transport system must be considered an inducible system. These data parallel the results of Schaefer (76) in E. coli cells regarding the active transport of B-glucosides.

### Inhibitors of uptake of lactose and of a-MG in lactose-induced cells

Hoffee et al. (39) have shown that glucose and a-MG are substrates of the glucose permease system which also has a high affinity for B-MG. Schaefer (76) confirmed these results with studies on the uptake of TEG in E. coli cells.

In A. faecalis the uptake of a-MG into whole cells is inhibited by glucose and, to a lesser extent, by cellobiose (Fig. 6). Lactose uptake in Alcaligenes is competitively inhibited by salicin and by cellobiose; a-MG and B-MG have no effect on lactose uptake (Fig. 7). These data suggest that the carbohydrate transport system follows a pattern similar to that in E. coli.

### The effect of osmotic shock on the uptake of carbohydrates

Kundig et al. (57) reported that whole cells of E.

Figure 5. Uptake of  $^{14}\text{C}$ -a-MG and  $^{14}\text{C}$ -lactose into uninduced *A. faecalis* cells. Cells were grown on basal salts medium containing sodium succinate (2%) as carbon source. Initial concentration of carbohydrates in each flask was  $^{14}\text{C}$ -a-MG,  $1.5 \times 10^{-5}$  M;  $^{14}\text{C}$ -lactose,  $1.2 \times 10^{-5}$  M. Symbols: ●-●, a-MG; ○-○, lactose.

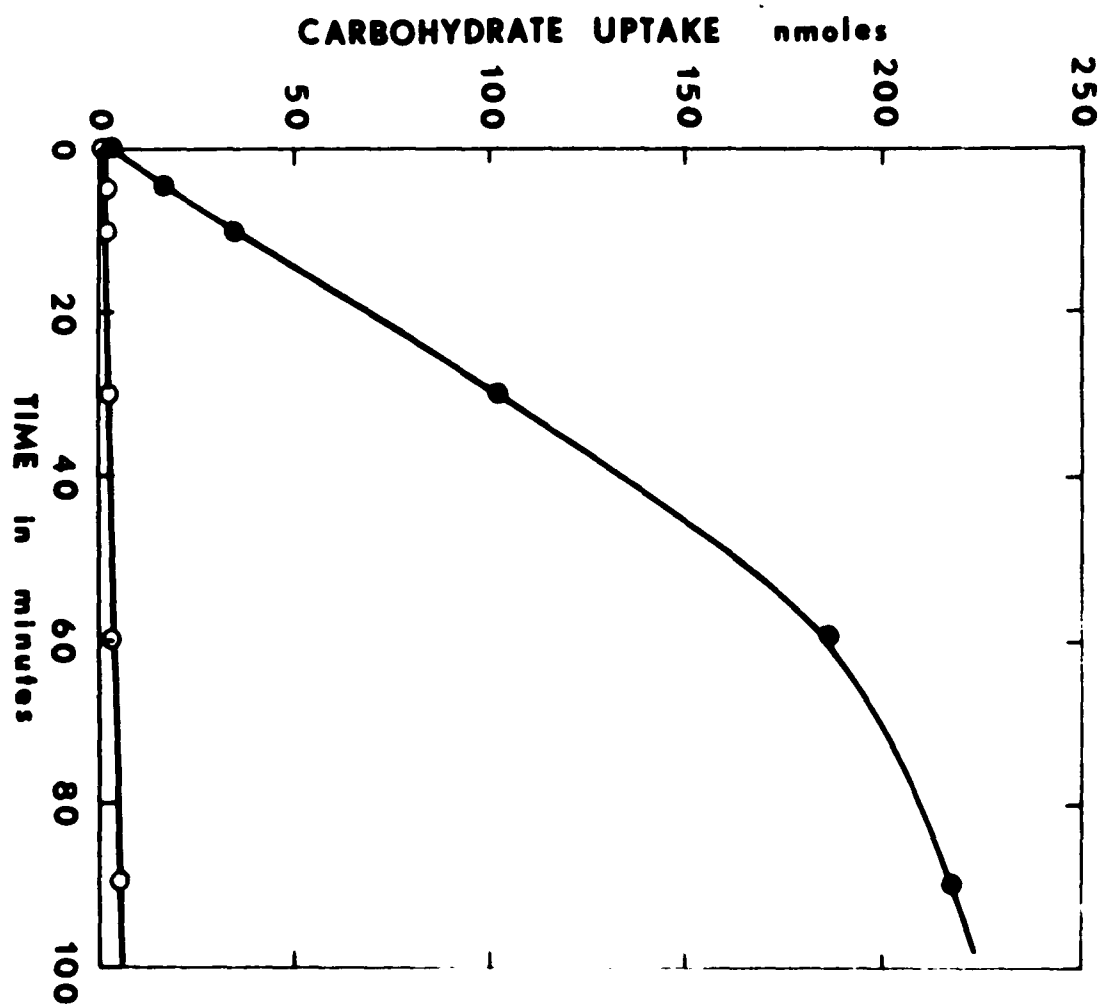


Figure 6. Inhibition of  $^{14}\text{C}$ -a-MG uptake in lactose-induced cells. Concentration of each inhibitor was  $1 \times 10^{-3}$  M.

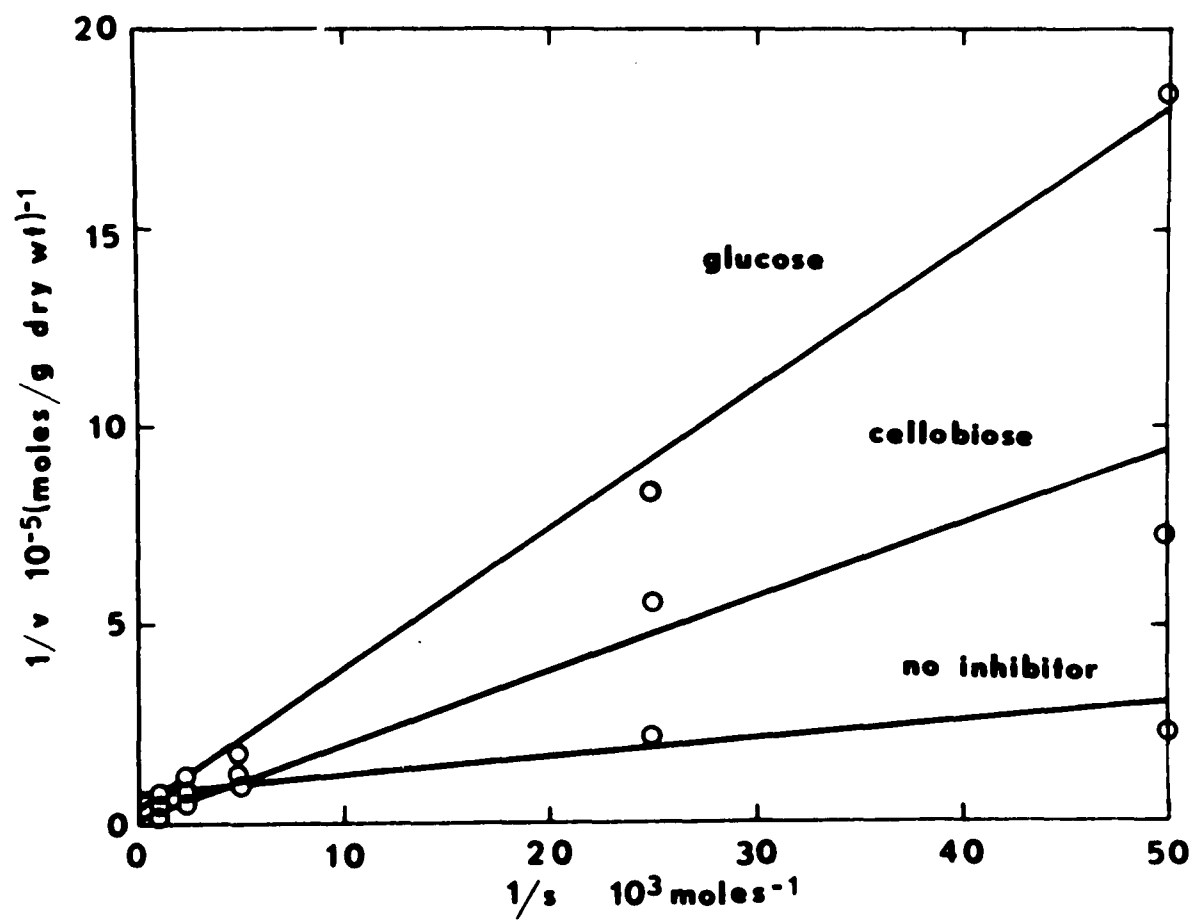
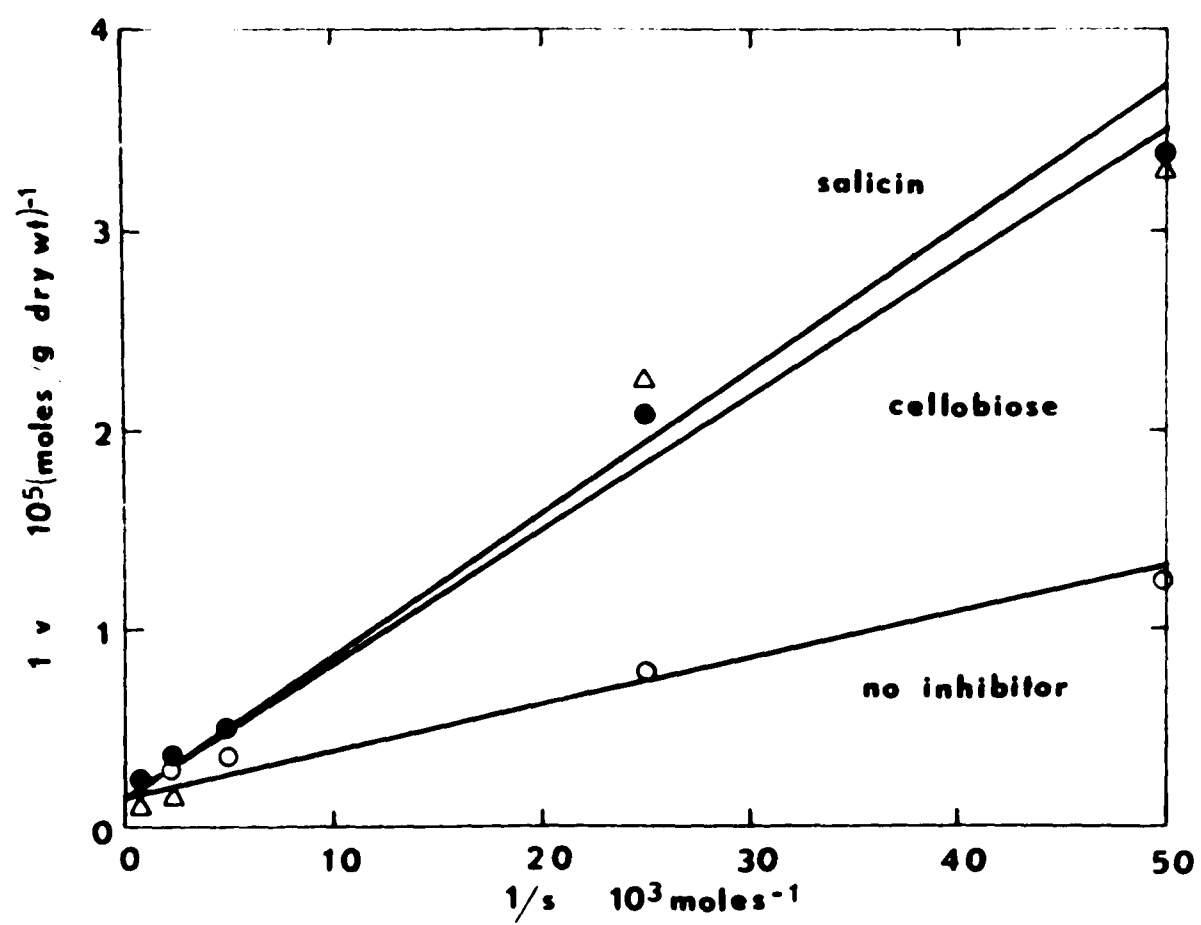




Figure 7. Inhibition of  $^{14}\text{C}$ -lactose uptake in lactose-induced cells. Concentration of each inhibitor was  $1 \times 10^{-3}$  M.



coli lost the ability to transport a-MG and TMG when subjected to the osmotic shock procedure of Nossal and Heppel (69). The effect of osmotic shock on carbohydrate transport in Alcaligenes is shown in Fig. 8 and 9. Lactose-induced cells which had been subjected to osmotic shock treatment lost the ability to actively transport lactose (Fig. 8). Lactose uptake in shocked cells is linear, in contrast to uptake in untreated cells which followed saturation kinetics. After 90 minutes, cells which had undergone osmotic shock treatment accumulated 24% of the amount of a-MG as had untreated cells (Fig. 9).

## II. Evidence for the Presence of Two Carbohydrases

### The effect of CTAB on whole cell activity

The effectiveness of the treatment of whole cells with CTAB prior to carbohydrase assay is illustrated in Table 3. Whole cells which had been treated with CTAB showed a four-fold increase in B-glucosidase specific activity over cells which had not been treated with the surfactant. Treatment of whole cells with CTAB effectively alters the permeability of the cell membrane to PNPG. CTAB-treated whole cells had 69% of the B-glucosidase specific activity as the crude cell extract obtained upon complete disruption of the cells by sonication.

The specific activity of B-galactosidase in whole cells which had not been treated with CTAB was 88% of the specific activity in the crude cell extract. B-glucosidase activity,

Figure 8. The effect of osmotic shock on the active uptake of  $^{14}\text{C}$ -lactose into lactose-induced cells. Initial concentration of lactose in each flask was  $1.2 \times 10^{-5}$  M. Symbols: ●-●, uptake in whole cells; ○-○, uptake in osmotically shocked cells.

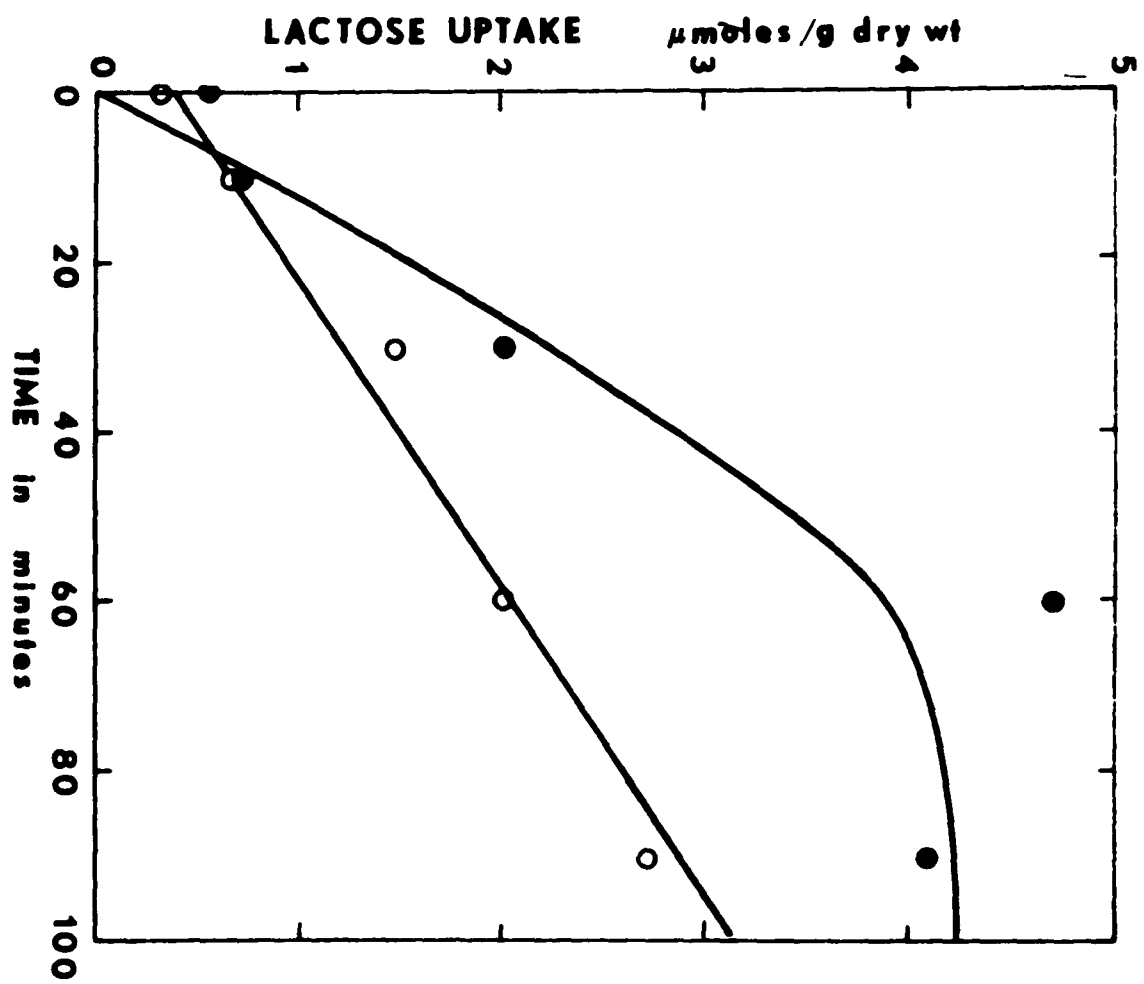


Figure 9. The effect of osmotic shock on the active uptake  $^{14}\text{C}$ -a-MG into lactose-induced cells. Initial concentration of a-MG in each flask was  $1.5 \times 10^{-5}$  M. Symbols: ●, uptake in whole cells; ○, uptake in osmotically shocked cells.

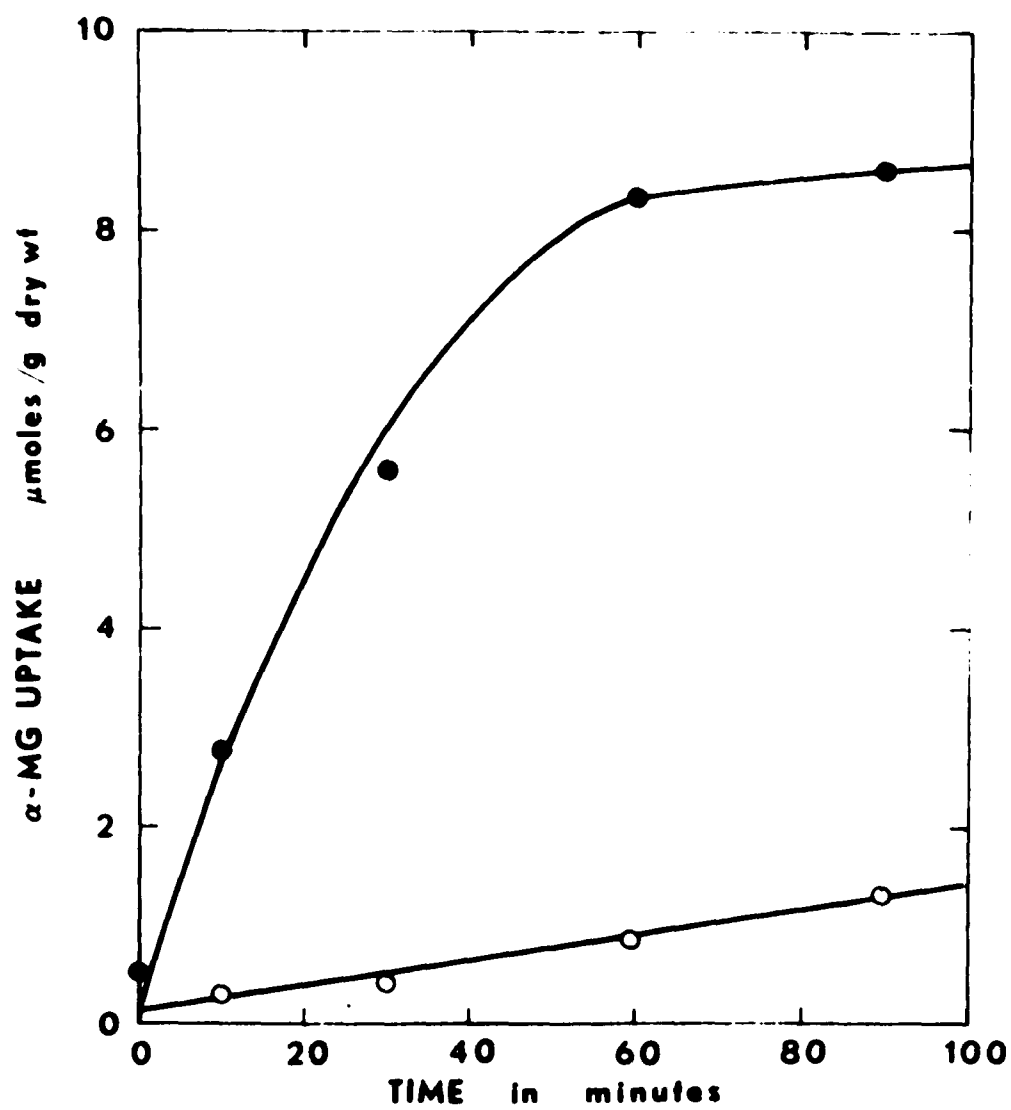


Table 3

Effect of CTAB on whole cell enzymatic  
activity in lactose-grown cells

Sample	Units/assay		Specific Activity (Units/g of protein)		<u>PNPGase</u> <u>ONPGase</u>
	<u>PNPGase</u> <sup>a</sup>	<u>ONPGase</u> <sup>b</sup>	<u>PNPGase</u>	<u>ONPGase</u>	
Whole cells without CTAB	0.0743	0.1105	19.1	28.3	0.7
Whole cells + CTAB	0.3156	0.0895	80.9	22.9	3.5
Crude cell extract <sup>c</sup>	0.4668	0.1289	116.7	32.2	3.6

<sup>a</sup> PNPGase: B-glucosidase

<sup>b</sup> ONPGase: B-galactosidase

<sup>c</sup> Whole cells were suspended in 0.1 M sodium phosphate buffer, pH 6.5 and sonicated for 7.5 minutes; the resulting mixture was centrifuged at 40,000 g for 30 minutes. The supernatant from this centrifugation is designated "crude cell extract."



however, was only 16% of that found in the cell extract. These data provide further evidence for the presence of a transport system for B-glucosides in addition to a B-galactoside transport system. ONPG freely traverses the membrane barrier in lactose-grown cells, but only low levels of PNPG are transported and subsequently hydrolyzed in cells which have not been grown on B-glucosides.

#### Induction of B-glucosidase and B-galactosidase in whole cells grown on different carbon sources

The organism produced different levels of B-glucosidase and B-galactosidase when the cells were induced by various carbohydrates (Table 4). The ratio of B-glucosidase specific activity to B-galactosidase specific activity in cells induced by cellobiose was 4:1. The ratio in lactose-induced cells was 2.8:1, and cells induced with B-MG produced a B-glucosidase to B-galactosidase ratio of 3.2:1. Salicin, an aryl B-glucoside, induced high levels of B-glucosidase but failed to increase B-galactosidase specific activity above that found in cells grown in the absence of inducer; the ratio of activities in salicin-induced cells was 7.5:1, indicating the possible presence of two distinct carbohydrases.

#### Analysis of mutant strains

Lactose mutants of Alcaligenes were assayed for B-glucosidase and B-galactosidase activities (Table 5). Levels of B-glucosidase and B-galactosidase in several of

Table 4

Levels of B-glucosidase and B-galactosidase in whole  
cells grown on different carbon sources<sup>a</sup>

Substrate	Specific Activity (units/g of protein)		<u>B-glucosidase</u> <u>B-galactosidase</u>
	B-glucosidase	B-galactosidase	
Cellobiose	97.0	24.0	4.0
Lactose	45.0	16.0	2.8
B-Methyl-glucoside	51.0	16.0	3.2
Galactose	32.0	10.0	3.2
Salicin	60.0	8.0	7.5
None	36.0	10.0	3.6

<sup>a</sup> Organisms were grown on basal salts medium containing 0.5% inducer.

Table 5

Levels of B-glucosidase and B-galactosidase in wild-type and mutant strains of Alcaligenes grown on cellobiose and on lactose<sup>a</sup>

Strain	Inducer	Specific Activity		<u>B-glucosidase</u> B-galactosidase
		B-glucosidase	B-galactosidase	
Wild-Type	Cellobiose	30.8	15.2	2.0
	Lactose	55.9	20.2	2.8
41 CL	Cellobiose	16.3	15.7	1.0
	Lactose	9.3	10.5	0.9
88 C	Cellobiose	30.3	46.5	0.7
	Lactose	24.0	17.1	1.4
91 BL	Cellobiose	39.5	15.7	2.5
	Lactose	60.5	18.9	3.2
202 BL	Cellobiose	36.0	14.4	2.5
	Lactose	59.0	16.5	3.6

<sup>a</sup> Cultures were grown in basal salts medium plus 0.5% casamino acids and 0.1% inducer. Cells were harvested, washed, suspended in 0.1 M sodium phosphate buffer, pH 6.5, and assayed for B-glucosidase, B-galactosidase and protein.

the mutants tested differed significantly from wild-type specific activity induced by the same carbohydrate. The ratio of the specific activities were also altered in several mutant strains.

The results of the mutant analyses indicate several possible mutational sites and support the hypothesis that more than one carbohydrase is present in Alcaligenes cells. Isolate 41CL produced levels of B-glucosidase lower than those of the wild-type when grown on both lactose and cellobiose. Furthermore, lactose-grown cells had lowered B-galactosidase levels. Since only one activity is affected primarily and the B-glucosidase:B-galactosidase ratio for both inducers is almost the same, this mutant most probably has an altered enzyme which is responsible for a portion of the B-glucosidase activity in the cell; a second enzyme still retains its original activity.

Mutant 88C could be called a "regulatory" mutant, because cellobiose appears to be a much more efficient inducer for both activities than lactose. The regulatory site for the induction of one enzyme activity only is affected because the B-glucosidase:B-galactosidase ratio for lactose-grown cells is similar to those ratios found in 41CL.

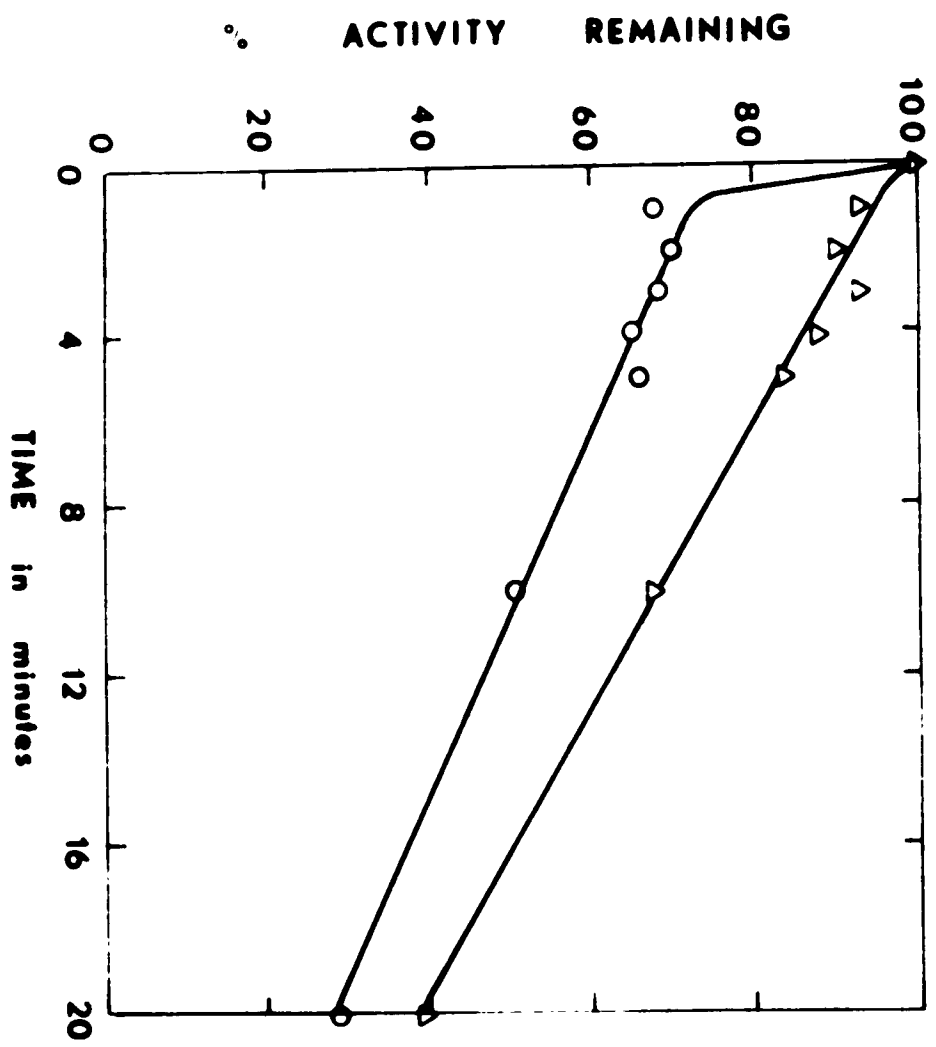
A third class of mutants is represented by 91BL and 202BL. Both of these strains were unable to grow on lactose-basal salts agar, but both strains show wild-type

levels of carbohydrase activity. These cultures are probably permease or carbohydrate transport mutants, since they are "cryptic" for B-glucoside and B-galactoside hydrolysis. Low levels of constitutive glycoside permease probably exist in these cells accounting for the induction of the carbohydrase.

#### Heat inactivation of crude cell extract

Studies on the heat inactivation of B-glucosidase and B-galactosidase activities at 55 C in crude cell extracts are summarized in Fig. 10. The heat inactivation curves for both activities exhibit bimodal kinetics. B-glucosidase and B-galactosidase activities decrease rapidly during the first minute of heat treatment. The decline in both activities then becomes more gradual. B-glucosidase activity decreased 30% during the first minute at 55 C; only 29% of the original activity remained after 20 minutes of heat treatment. Approximately 7% of the B-galactosidase activity was lost after one minute. 39% of the activity in untreated extract remained after 20 minutes at 55 C. Purified B-glucosidase from Alcaligenes is readily inactivated at temperatures above 55 C (27). However, the purified enzyme is relatively heat stable at 55 C or below. The bimodal kinetics of heat inactivation of the crude cell extract result when a heat-labile carbohydrase is inactivated rapidly at 55 C, followed by the gradual inactivation of the B-glucosidase.

Figure 10. Heat inactivation of B-glucosidase and B-galactosidase activities in a cell extract of lactose-induced cells. Symbols: O-O, B-glucosidase;  $\Delta$ , B-galactosidase.



### The release of carbohydrase activity from cells subjected to osmotic shock

Osmotically shocked cells exhibit loss of both B-glucosidase and B-galactosidase activities (Table 6). Shock protein was found to have a B-glucosidase specific activity 6.6 times the specific activity found in whole cells, while B-galactosidase specific activity was 26.3 times that of whole cells. The ratio of B-glucosidase activity to B-galactosidase activity was 1.09 in shock protein, whereas the ratio of the two activities in whole cells was 4.4.

### The effect of sonication on osmotically shocked cells

Osmotically shocked cells release B-glucosidase and B-galactosidase activities upon sonication (Fig. 11). Whole cells are completely disrupted by sonication for 7.5 minutes. Maximal release of enzymatic activities from osmotically shocked cells is obtained after 60 seconds of sonication. The specific activities of B-glucosidase and B-galactosidase released from the cells after 60 seconds of sonication are 11.0 and 2.1 units per gram, respectively, and the ratio of B-glucosidase to B-galactosidase is 5.2:1. B-glucosidase activity in the whole cells from which this preparation was made was 36.3 units per gram; the B-galactosidase specific activity was 8.0.

### Elution profiles of gel filtration columns

The profile of the elution pattern obtained upon gel filtration of concentrated cell extract from lactose-induced



Table 6

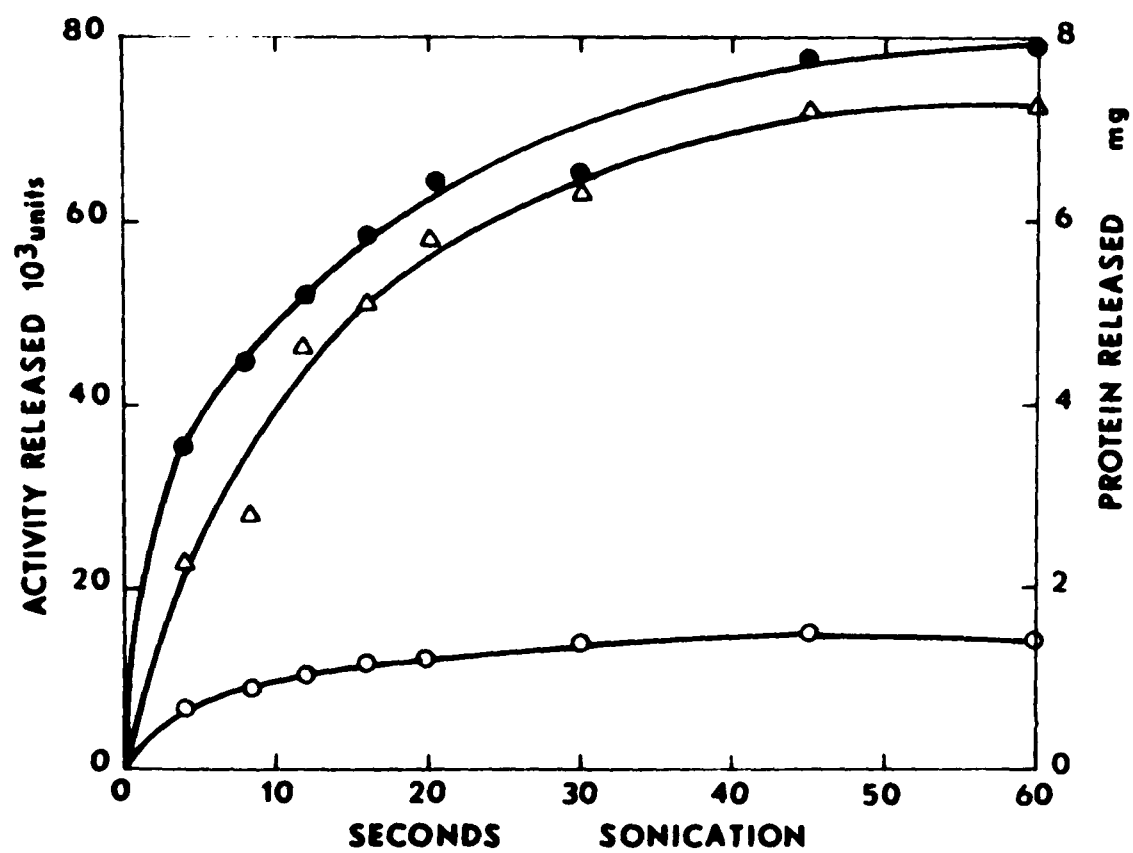
Release of carbohydrase activity from lactose-induced Alcaligenes during osmotic shock

Treatment	Units activity		(mg/assay)	Specific Activity		
	PNPGase <sup>a</sup>	ONPGase <sup>b</sup>		(units/gram) PNPGase	ONPGase	<u>PNPGase</u> <u>ONPGase</u>
Whole cells (0.5 ml/ assay)	0.1330	0.0305	3.050	43.6	10.0	4.4
Shocked cells (0.5 ml/ assay)	0.0716	0.0236	3.150	22.7	7.5	3.0
Shock protein	0.0069	0.0063	0.024	287.5	262.5	1.1

<sup>a</sup> PNPGase: B-glucosidase

<sup>b</sup> ONPGase: B-galactosidase

Figure 11. The release of enzymatic activity and of protein from osmotically shocked cells during sonication. Osmotically shocked cells were suspended in 0.1 M sodium phosphate buffer, pH 6.5, and sonicated. Samples were removed at various time intervals, centrifuged and assayed for enzymatic activity and protein content. Symbols: ●-●, B-glucosidase; O-O, B-galactosidase; Δ-Δ, protein.



cells is shown in Fig. 12. Two peaks of enzymatic activity are eluted from Sephadex G-100. Activity peaks of B-glucosidase and B-galactosidase were found in fractions 41 and 50. The first active peak eluted with the major protein peak and had a B-glucosidase to B-galactosidase ratio of 2.5. The second peak eluted from the column had a B-glucosidase to B-galactosidase ratio of 4.6:1; this activity was stable to lyophilization with 1% inositol, as was the previously purified B-glucosidase.

In order to determine if the B-glucosidase activity induced by salicin was a third enzyme having salicinase or aryl B-glucosidase activity, a concentrated extract of osmotically shocked cells was prepared from salicin-induced cells. The elution pattern from gel filtration of this extract showed only two peaks of B-glucosidase activity (Fig. 13).

The first enzymatically active peak obtained from induced cells appeared to be particulate in nature. In order to test this hypothesis, the concentrated extract was centrifuged at 100,000 x g on a Beckman Model L2-65B ultracentrifuge for 30 min. The supernatant fluid was decanted and subjected to gel filtration. The elution pattern obtained is shown in Fig. 14.

Enzymatic activity was found in one peak corresponding to B-glucosidase; a reduction in the size of the major protein peak relative to B-glucosidase activity was also observed. It may be concluded from these observations

Figure 12. Elution profile on gel filtration (Sephadex G-100) of concentrated extract of osmotically shocked cells induced by lactose. Symbols: O-O, absorbance; ●-●, B-glucosidase; Δ-Δ, B-galactosidase.

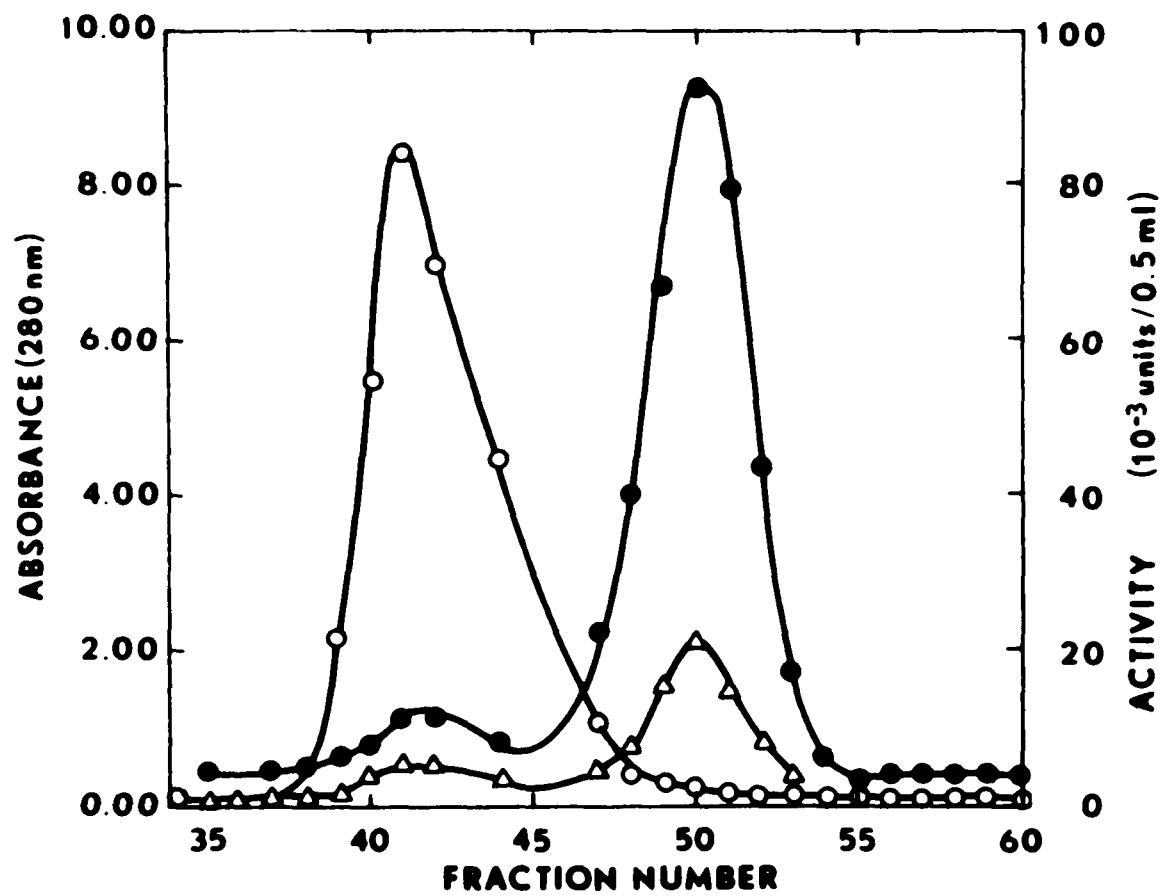


Figure 13. Elution profile on gel filtration (Sephadex G-100) of concentrated extract of osmotically shocked cells induced by salicin. Symbols: O-O, absorbance at 280 nm; ●-●, B-glucosidase.

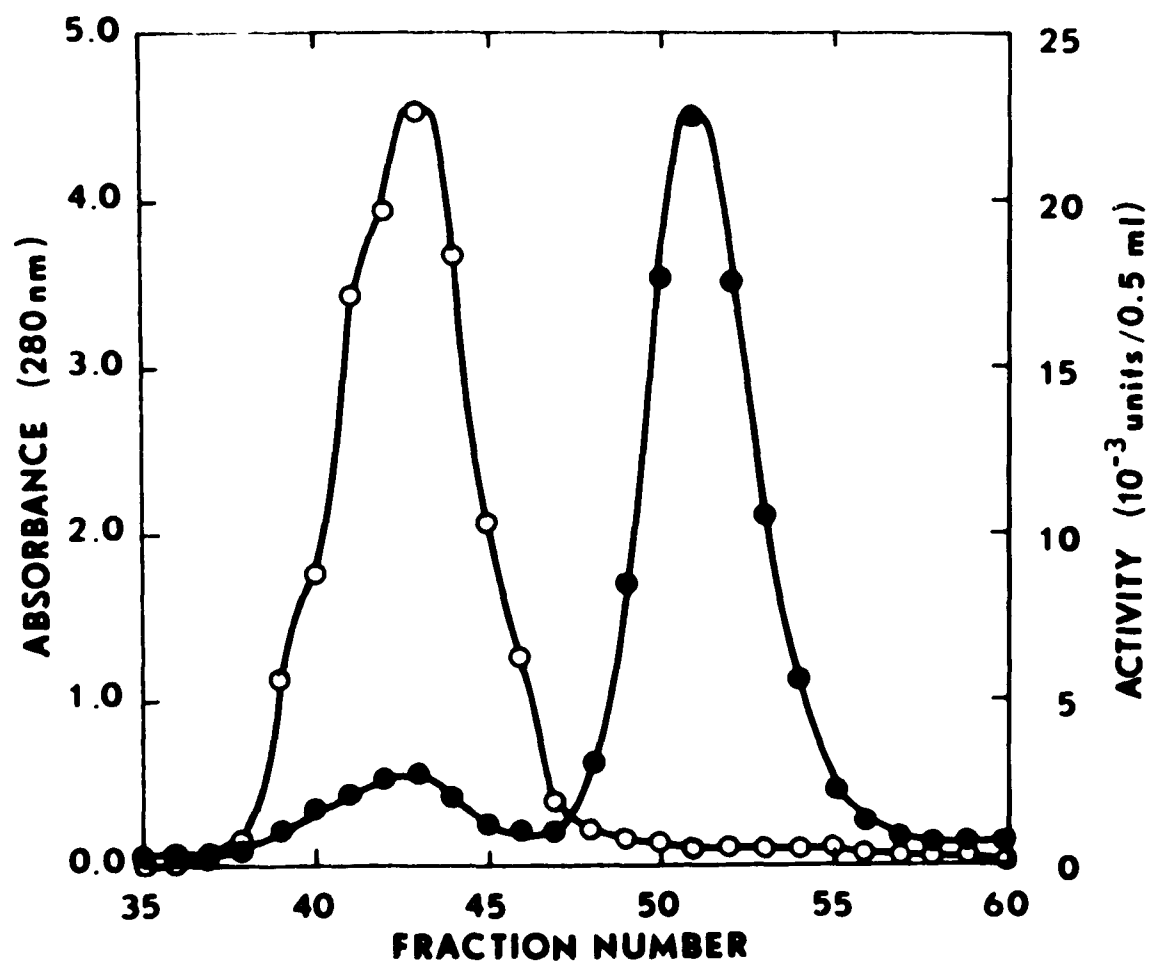
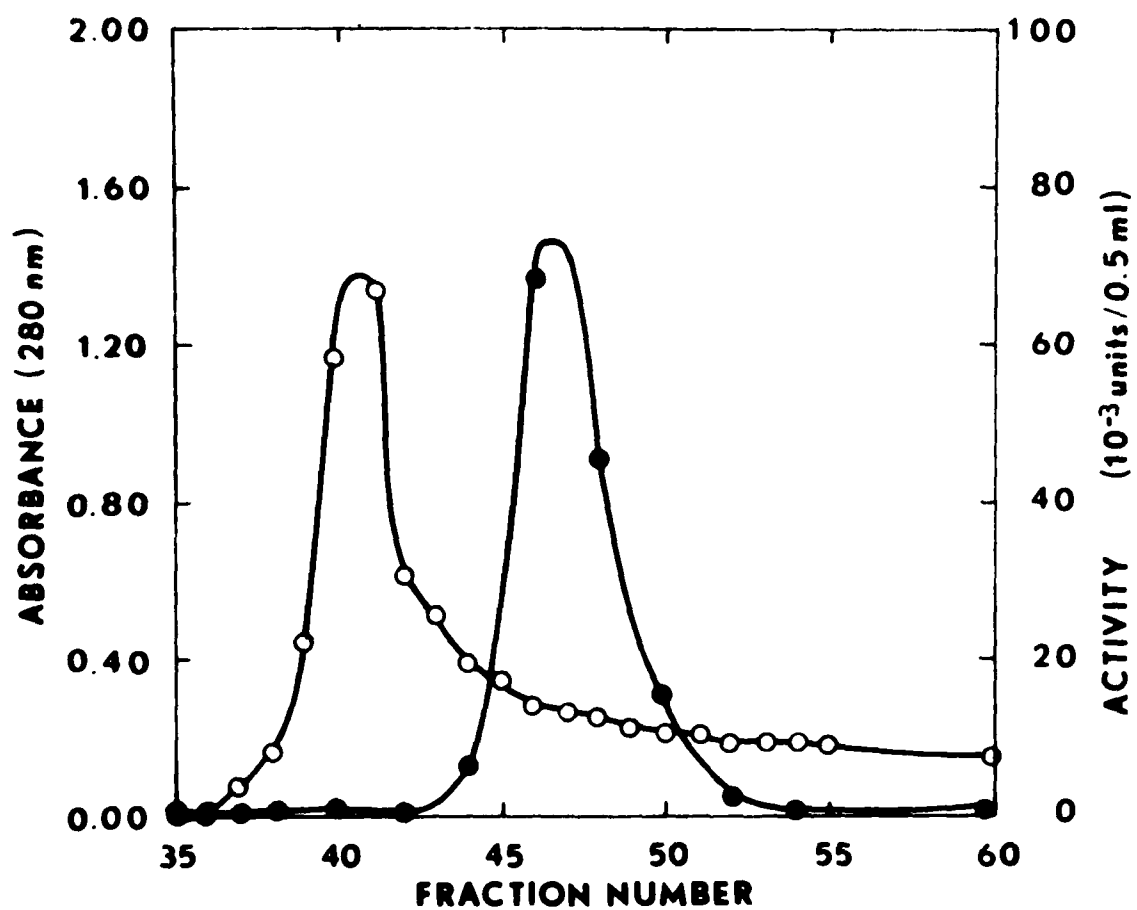




Figure 14. Elution profile on gel filtration (Sephadex G-100) of concentrated extract of osmotically shocked lactose-induced cells after high speed centrifugation. Concentrated cell extract was centrifuged at 100,000 x g for 30 minutes. Symbols: O-O, absorbance at 280 nm; ●-●, B-glucosidase.



that one carbohydrase demonstrated by gel filtration is particulate in nature, while the second existed as a soluble protein.

### III. Purification and Composition of B-glucosidase

#### Purification of B-glucosidase

A summary of the procedure followed in the purification of B-glucosidase is shown in Table 7. The enzyme was purified 24.5-fold with a recovery of about 70% of the initial enzyme activity.

#### Preparative polyacrylamide gel electrophoresis

The peak of B-glucosidase activity eluted from the preparative polyacrylamide gel electrophoresis column was found to be homogeneous (Fig. 15). A total of 3.3 mg of protein was recovered which had a specific activity of 55,735 units/g.

#### Amino acid analysis of B-glucosidase

The amino acid composition of B-glucosidase is shown in Table 8. Values for the number of residues of arginine, cysteine, and tryptophan were not obtained. A molecular weight of 120,000 to 125,000 was estimated previously by Han (27) using a sucrose density gradient.

Table 7

## Purification of B-glucosidase

Treatment	Total volume (ml)	Total enzyme (units)	Total protein (mg)	Specific activity (units/g)	% Recovery	Purific action
Whole cells	40	19.09	263.0	80.9	100	---
Supernatant fluid of sonicated, osmotically shocked cells	20	15.49	188.0	82.4	81	1.0X
Sephadex (G-100) second peak	103.2	13.29	6.7	1938.0	70	24.5X

**FIGURE 15.** Polyacrylamide gel electrophoresis of purified B-glucosidase. Concentrated extract of osmotically shocked cells was centrifuged at 100,00 x g and loaded onto a preparative polyacrylamide gel for electrophoresis. Active fractions were pooled and concentrated for amino acid analysis.

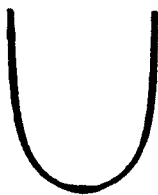


Table 8

Amino acid composition of A.  
faecalis B-glucosidase<sup>a</sup>

Amino Acid	Residues
Lysine	35.4
Histidine	18.0
Aspartic acid	80.2
Threonine	43.1
Serine	40.8
Glutamic acid	87.1
Proline	52.2
Glycine	79.3
Alanine	71.8
Valine	61.9
Methionine	12.3
Isoleucine	39.3
Leucine	65.2
Tyrosine	12.0
Phenylalanine	24.9

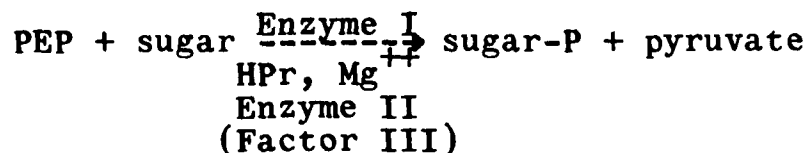
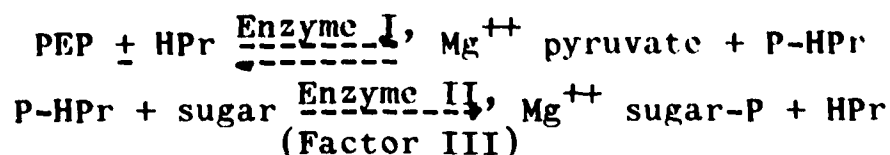
<sup>a</sup> Expressed as residues per 120,000 molecular weight.

## DISCUSSION

Egan and Morse (20) isolated a mutant of S. aureus which had simultaneously lost the ability to utilize a number of carbohydrates but retained the ability to utilize glucose. They later concluded that transport in Staphylococcus involved permeases specific for various carbohydrates and a single carrier common to the transport of all sugars (21, 22). A single mutation had resulted in the loss of the ability of the cell to transport eight carbohydrates, and several carbohydrates competitively inhibited the uptake of other sugars (21). Carbohydrates were found to be accumulated by this organism as the phosphorylated derivatives (33). Furthermore, hydrolysis of lactose within the staphylococcal cell requires phosphorylation of the carbohydrate (33, 34).

Phosphorylation of lactose is thought to occur during the passage of the molecule through the staphylococcal membrane via a PEP-linked phosphotransferase system (24, 32, 35, 50, 81). Evidence for a phosphotransferase system also has been found in Salmonella typhimurium (57), Aerobacter aerogenes (75, 86), and E. coli. The overall phosphotransferase system is a series of individual reactions:





The phosphotransferase system in E. coli and S. typhimurium is composed of two enzymes, I and II, and a heat stable protein HPr (57). Factor III, a fourth component has been isolated from S. aureus (82). Enzyme I and HPr are found in the soluble fraction of the cell (57).

The active transport and utilization of B-glucosides in E. coli has been studied and described in detail (76, 77). While wild-type E. coli strains do not split B-glucosides, inducible mutants can be isolated which do so (76). The mutation B-gl<sup>-</sup> to B-gl<sup>+</sup> is characterized by the appearance of an inducible permease and an aryl B-glucosidase; aryl B-glucosides serve as better inducers than alkyl B-glucosides (76). <sup>14</sup>C-TEG was taken up by two different permeases: (a) permease I was constitutive and its characteristics were similar to those of the glucose permease system; (b) permease II was inducible and could be found only in induced B-gl<sup>+</sup> cells (76). Schaeffler and Maas (77) demonstrated at least three mutational sites, representing three distinct loci associated with the utilization of B-glucosides in E. coli: (a) a structural gene of

an aryl B-glucoside-splitting enzyme; (b) either a structural gene for a B-glucoside permease or a regulatory gene; and (c) a pleiotropic locus either of regulator or of operator type (77).

The carbohydrate transport systems in Alcaligenes faecalis (ATCC 21400) are not unlike those transport systems described above (33, 39, 77). Carbohydrates are transported into the cell via at least four distinct transport systems. The lactose transport system is inducible and has no affinity for B-MG, a-MG or PNPG. A constitutive glucose transport system is responsible for the transport of a-MG and B-MG. Competitive inhibition of both the lactose and glucose transport mechanisms by cellobiose and of the lactose transport system by salicin indicate specificity for, respectively, the B-1,4 linkage and the glucose moiety. B-glucosides may also be transported via a B-glucoside transport system such as that found in E. coli (76). A fourth system of carbohydrate transport may be proposed which is specific for disaccharides containing the a-1,4 linkage, since sucrose (a-D-glucopyranosyl-B-D-fructofuranoside) and maltose (4-0-a-D-glucopyranosyl-D-glucopyranoside) are both transported by whole cells.

The effect of osmotic shock on lactose transport in E. coli cells was reported by Kundig et al. (58). The loss of the ability to transport carbohydrates upon

osmotic shock treatment is attributed to the loss of certain of the so-called pericytoplasmic proteins (36), including HPr. These proteins have been released by gentle treatment from E. coli and closely related Gram-negative bacteria. Certain cell wall characteristics may be responsible for the relative ease with which these proteins are released. Cold osmotic shock, as described by Nossal and Heppel (69), failed to effect the release of protein from Alcaligenes. However, partial digestion of the cell wall in Alcaligenes by lysozyme makes this organism susceptible to osmotic shock with the loss of certain transport properties and the release of B-glucosidase and B-galactosidase activities.

It may be noted here that carbohydrate-binding activity (5) in shock protein from Alcaligenes cells could not be demonstrated and that efforts to reconstitute active transport in osmotically shocked cells by the addition of concentrated shock protein were unsuccessful. Standardization of the osmotic shock procedure, as measured by the release of protein labelled with  $^{14}\text{C}$ -leucine, was also difficult: values for the release of protein into shock fluid ranged from 12 to 21% of the total cell protein. These data are not surprising since Kundig et al. (57, 58) have reported wide variation in their results. Of 26 shocked cell preparations, transport was reconstituted in 11 by the addition of purified HPr from shock fluid.

The rate-limiting step in the utilization of carbohydrate is the transport of the sugar into the cell. Ulitzer (87) showed that permeaseless E. coli cells transported B-galactosides after treatment with CTAB, a cationic detergent. Concentrations of CTAB used for whole cell assays in Alcaligenes were about ten times those used to effect the transport of galactosides into E. coli cells. CTAB acts specifically to make Alcaligenes cells permeable to B-glucosides in cells which have not been induced for their transport. Furthermore, at the concentrations used in the present investigation, the detergent has apparently no adverse effects on those carbohydrate permeases present in the induced system.

Clearly, the indirect evidence for the presence of two carbohydrases in Alcaligenes is convincing. Although some investigators have failed to separate B-glucosidase activity from B-galactosidase activity (91) and maintain that these two carbohydrase activities cannot be separated, two carbohydrases of apparently different specificities are present in this organism, and they can readily be separated by physical and chemical means.

The specificity of B-glucosidase has been previously examined (27). The enzyme shows a marked preference for the B-glucosidic linkage, but it hydrolyzes PNPG and ONPG in a ratio of 4.6:1. The second carbohydrase which has been demonstrated in this organism has a B-glucosidase

to B-galactosidase ratio of 2.5:1. Further definitive studies on the second enzyme with regard to its substrate specificity are necessary. However, it may be assumed that it has a greater hydrolytic activity toward the B-(1,4)-galactosidic linkage than does the B-glucosidase, since the B-glucosidase has very little activity toward lactose. Alcaligenes utilizes lactose as sole carbon source, and the very low activity of B-glucosidase toward this substrate cannot account for the ability of the organism to efficiently metabolize lactose. The activity of carbohydrase II could thus account for the growth of the organism on the carbon source lactose.

The relationship between the two carbohydrases which have been demonstrated in Alcaligenes is at this time unclear. B-glucosidase has been shown to be soluble and has been purified to homogeneity. Carbohydrase II, on the other hand is particulate in nature. This could account for the failure to recover two activities from diethylaminoethyl (DEAE) cellulose. It is plausible to assume that the second carbohydrase is membrane-bound as is the B-glucosidase of E. coli (76). CTAB-treatment of whole cells slightly decreased the B-galactosidase activity below levels found in untreated cells. This decrease may be due to disruption of the enzyme-membrane complex by the detergent resulting in the loss of enzymatic activity.

Alcaligenes faecalis is described in Bergey's Manual

(6) as not utilizing carbohydrates. However, the type culture (ATCC 8750) as well as the strain used in the present studies both are able to grow on lactose as sole carbon source although quite low cell yields were obtained (27). However, both strains were found to be lactose-negative in the peptone medium commonly used in the test for the utilization of carbohydrates. The ability of an organism to utilize a specific carbohydrate is therefore dependent upon not only its genetic capability to do so, but also upon the presence or absence of other essential growth factors in the medium. Cell yields of Alcaligenes were increased more than two-fold over those previously obtained on lactose medium by the addition of 2% sodium succinate and 0.1% yeast extract.

The mechanisms for carbohydrate utilization are present in Alcaligenes. A number of carbohydrates are transported and accumulated in whole cells. Furthermore, the hydrolytic enzymes necessary for the breakdown of B-glucosides and B-galactosides are inducible in the organism. A single mutational event could not be responsible for the acquisition of all of the characteristics demonstrated by the present study by Alcaligenes faecalis (ATCC 21400). Other members of the genus Alcaligenes may possess some or all of these transport and carbohydrase properties. Considerable taxonomic significance would therefore be attached to the demonstration of similar

characteristics in other species in the genus Alcaligenes.

## LITERATURE CITED

1. Abrahams, H. E., and D. Robinson. 1969. B-D-Glucosidases and related enzymic activities in pig kidney. *Biochem. J.* 111: 749-755.
2. Adams, E. Nomograph distributed by California Corporation for Biochemical Research. 3625 Medford St., Los Angeles, California.
3. Agrawal, K. M. L., and O. P. Bahl. 1968. Glycosidases of Phaseolus vulgaris. II. Isolation and general properties. *J. Biol. Chem.* 243: 103-111.
4. Aizawa, K. 1939. Studien Über Carbohydase I. Die fermentative Hydrolyse der p-Nitrophenylglucoside. *J. Biochem. (Tokyo)* 30: 89-100.
5. Boos, W. 1969. The galactose binding protein and its relationship to the B-methylgalactoside permease from Escherichia coli. *European J. Biochem.* 10: 66-73.
6. Breed, R. S., E. G. D. Murray and N. R. Smith. 1957. *Bergey's Manual of determinative bacteriology*, 7th ed. The Williams and Wilkins Co., Baltimore.
7. Bucht, B. and K.-E. Eriksson. 1969. Extracellular enzyme system utilized by the rot fungus Stereum sanguinolentum for the breakdown of cellulose. IV. Separation of cellobiase and aryl B-glucosidase activities. *Arch. Biochem. Biophys.* 129 : 416-420.
8. Cirillo, V. P. 1961. The transport of non-fermentable sugars across the yeast cell membrane, p. 343-351. In: Kleinzeller, A. and A. Kotyk (ed.) *Membrane transport and metabolism. A symposium.* Academic Press, Inc., New York.
9. Cohen, G. N. and J. Monod. 1957. Bacterial permeases. *Bacteriol. Rev.* 21: 169-194.
10. Cohn, M., and J. Monod. 1951. Purification et proprietes de la B-galactosidase (lactase) d'Escherichia coli. *Biochim. Biophys. Acta* 7: 153-174.
11. Conchie, J. 1954. B-glucosidase from rumen liquor: Preparation, assay and kinetics of action. *Biochem. J.* 58: 552-560.



12. Conchie, J., A. L. Gelman and G. A. Levvy. 1967. Inhibition of glycosidases by aldonolactones of corresponding configuration: The C-4- and C-6-specificity of B-glucosidase and B-galactosidase. *Biochem. J.* 103: 609-615.
13. Dahlqvist, A. 1961. Pig intestinal B-glucosidase activity. I. Relation to B-galactosidase (lactase). *Biochim. Biophys. Acta* 50: 55-61.
14. Dance, N., R. G. Price, D. Robinson and J. L. Stirling. 1969. B-galactosidase, B-glucosidase and N-acetyl-B-glucosaminidase in human kidney. *Clin. Chim. Acta* 24: 189-197.
15. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121: 404-427.
16. Dean, A. C. R. and P. J. Rodgers. 1969. Steady state levels of dehydrogenases and a- and B-glucosidases in Klebsiella aerogenes. *J. Gen. Microbiol.* 57: 169-178.
17. Duerksen, J. D. and H. Halvorson. 1958. Purification and properties of an inducible B-glucosidase of yeast. *J. Biol. Chem.* 233: 1113-1120.
18. Eberhart, B. M. and R. S. Beck. 1970. Localization of the B-glucosidases in Neurospora crassa. *J. Bacteriol.* 101: 408-417.
19. Eberhart, B., D. F. Cross and L. R. Chase. 1964. B-glucosidase system of Neurospora crassa. I. B-glucosidase and cellulase activities of mutant and wild-type strains. *J. Bacteriol.* 87: 761-770.
20. Egan, J. B. and M. L. Morse. 1965. Carbohydrate transport in Staphylococcus aureus. I. Genetic and biochemical analysis of a pleiotropic mutant. *Biochim. Biophys. Acta* 97: 310-319.
21. Egan, J. B. and M. L. Morse. 1965. Carbohydrate transport in Staphylococcus aureus. II. Characterization of the defect of a pleiotropic transport mutant. *Biochim. Biophys. Acta* 109: 172-183.

22. Egan, J. B. and M. L. Morse. 1966. Carbohydrate transport in Staphylococcus aureus. III. Studies of the transport process. Biochim. Biophys. Acta 112: 63-73.
23. Fields, K. L. and S. E. Luria. 1969. Effects of colicins E1 and K on transport systems. J. Bacteriol. 97: 57-63.
24. Fraenkel, D. G. 1968. The accululation of glucose 6-phosphate from glucose and its effect in an Escherichia coli mutant lacking phosphoglucose isomerase and glucose 6-phosphate dehydrogenase. J. Biol. Chem. 243: 6451-6457.
25. Fox, C. F. and E. P. Kennedy. 1965. Specific labeling and partial purification of the M protein, a component of the B-galactoside transport system of Escherichia coli. Proc. Nat. Acad. Sci. 54: 891-899.
26. Haisman, D. R., D. J. Knight and M. J. Ellis. 1967. The electrophoretic separation of the B-glucosidases of almond "emulsin". Phytochemistry 6: 1501-1505.
27. Han, Y. W. 1969. Studies on the bacteria and bacterial enzymes involved in the degradation of cellulose. Ph.D. Dissertation.
28. Han, Y. W. and V. R. Srinivasan. 1968. Isolation and characterization of a cellulose utilizing bacterium. Appl. Microbiol. 16: 1140-1145.
29. Han, Y. W. and V. R. Srinivasan. 1969. Purification and characterization of B-glucosidase of Alcaligenes faecalis. J. Bacteriol. 100: 1355-1363.
30. Hash, J. H. and K. W. King. 1958. Some properties of an aryl B-glucosidase from culture filtrates of Myrothecium verrucaria. J. Biol. Chem. 232: 395-402.
31. Hash, J. H. and K. W. King. 1958. On the nature of B-glucosidases in Myrothecium verrucaria. J. Biol. Chem. 232: 381-393.

32. Hengstenberg, W. 1970. Solubilization of the membrane bound lactose specific component of the staphylococcal PEP-dependent phosphotransferase system. FEBS Letters 8: 277-280.
33. Hengstenberg, W., J. B. Egan and M. L. Morse. 1967. Carbohydrate transport in Staphylococcus aureus. V. The accumulation of phosphorylated carbohydrate derivatives and evidence for a new enzyme-splitting lactose phosphate. Biochemistry 58: 274-279.
34. Hengstenberg, W., J. B. Egan and M. L. Morse. 1968. Carbohydrate transport in Staphylococcus aureus. VI. The nature of the derivative accumulated. J. Biol. Chem. 243: 1881-1885.
35. Hengstenberg, W., W. K. Penberthy, K. L. Hill and M. L. Morse. 1969. Phosphotransferase system of Staphylococcus aureus. Its requirement for the accumulation and metabolism of galactosides. J. Bacteriol. 99: 383-387.
36. Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science. 156: 1451-1455.
37. Heyworth, R. and P. G. Walker. 1961. Identity of B-glucosidase and B-galactosidase in sweet-almond emulsin. Biochem J. 80: 44P.
38. Heyworth, R. and P. G. Walker. 1962. Almond-emulsin-B-D-glucosidase and B-D-galactosidase. Biochem. J. 83: 331-335.
39. Hoffee, P. E. Englesberg and F. Lamy. 1964. The glucose permease system in bacteria. Biochim. Biophys. Acta 79: 337-350.
40. Horikoshi, K. and T. Yonosuke. 1965. Studies on the spore coats of Aspergillus oryzae. II. Conidacoat bound B-glucosidase. Biochem. Biophys. Acta 101: 352-357.
41. Hughes, M. A. 1968. Studies on B-glucosidase production in cultured tissue of Trifolium repens L. J. Exp. Bot. 19: 52-63.

42. Jermyn, M. A. 1955. Fungal cellulases. IV. Production and purification of an extra-cellular B-glucosidase of Stachybotrys atra. Austral. J. Biol. Sci. 8: 541-562.
43. Jermyn, M. A. 1955. Fungal cellulases. VI. Substrate and inhibitor specificity of the B-glucosidase of Stachybotrys atra. Austral. J. Biol. Sci. 8: 577-602.
44. Jermyn, M. A. 1958. Fungal cellulases. VIII. Further observations on the B-glucosidase of Stachybotrys atra. Austral. J. Biol. Sci. 11: 114-126.
45. Jermyn, M. A. 1962 Fungal cellulases. X. Further purification of the B-glucosidase from Stachybotrys atra. Austral. J. Biol. Sci. 15: 769-786.
46. Kaback, H. R. 1970. Transport. Ann. Rev. of Biochem. 39: 561-598.
47. Kaplan, J. G. 1965. An inducible system for the hydrolysis and transport of B-glucosides in yeast. I. Characteristics of the B-glucosidase activity of intact and of lysed cells. J. Gen. Physiol. 48: 873-886.
48. Keegstra, K. and P. Albersheim. 1969. The involvement of glycosidases in the cell wall metabolism of suspension-cultured Acer pseudo-platanus cells. Plant Physiol. 45: 675-678.
49. Kennedy, E. P. 1970. The lactose permease system of Escherichia coli. p. 49-92. In: Beckwith, J. R. and D. Zipser (ed.). The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
50. Kennedy, E. P. and G. A. Scarborough. 1967. Mechanism of hydrolysis of o-nitrophenyl-B-galactoside in Staphylococcus aureus and its significance for theories of sugar transport. Proc. Natl. Acad. Sci. U. S. 58: 225-228.
51. Kepes, A. 1960. Etudes cinetiques sur la galactoside-permease d'Escherichia coli. Biochem. Biophys. Acta 40: 70-84.

52. Kepes, A. and G. N. Cohen. 1962. Permeation. p. 179-221. In: Gunsalus, I. C. and R. Y. Stanier (ed.). The bacteria. IV: The physiology of growth. Academic Press, New York, N. Y.
53. King, K. W. and R. M. Smibert. 1963. Distinctive properties of B-glucosidases and related enzymes derived from a commercial Aspergillus niger cellulase. Appl. Microbiol. 11: 315-319.
54. Koch, A. L. 1964. The role of permease in transport. Biochim. Biophys. Acta 79: 177-200.
55. Koch, A. L. 1967. Kinetics of permease catalyzed transport. J. Theoret. Biol. 14: 103-130.
56. Kooiman, P., P. A. Roelofsen and S. Swaris. 1953. Some properties of cellulase from Myrothecium verrucaria. Enzymologia 16: 237-246.
57. Kundig, W., S. Ghosh and S. Roseman. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Proc. Natl. Acad. Sci. U. S. 52: 1067-1074.
58. Kundig, W., F. D. Kundig, B. Anderson and S. Roseman. 1966. Restoration of active transport of glycosides in Escherichia coli by a component of a phosphotransferase system. J. Biol. Chem. 241: 3243-3246.
59. Landman, O. F. 1957. Properties and induction of B-galactosidase in Bacillus megaterium. Biochem. Biophys. Acta 23: 558-569.
60. Lederberg, J. 1950. The B-galactosidase of Escherichia coli, strain K-12. J. Bacteriol. 60: 381-392.
61. Lederberg, J. and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63: 399-406.
62. Lowery, O. H., N. Y. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.

63. Mahadeven, P. R. and B. Eberhart. 1964. Aryl-B-glucosidase of some Neurospora strains. Biochim. Biophys. Acta 90: 214-215.
64. Mao, C.-H. and L. Anderson. 1967. Cyanogenesis in Sorghum vulgare. III. Partial purification of two B-glucosidases from Sorghum tissues. Phytochemistry 6: 473-483.
65. Marchin, G. L. and J. D. Duerksen. 1968. Purification of B-glucosidase from Saccharomyces lactis strain Y-123. J. Bacteriol. 96: 1181-1186.
66. Mitchell, P. 1961. Approaches to the analysis of specific membrane transport. p. 581-603. In: Goodwin, T. W. and O. Lindberg (ed.). Biological structure and function. Proc. 1st IUB/IUBS Int. Symp. Vol. II. Academic Press, Inc., New York, N. Y.
67. Monod, J., G. Cohen-Bazire and M. Cohn. 1951. Sur la biosynthese de la B-galactosidase (lactase) chez Escherichia coli. La specificite de l'induction. Biochim. Biophys. Acta 7: 585-599.
68. Neu, H. C. and L. A. Heppel. 1964. The release of ribonuclease into the medium when E. coli cells are converted to spheroplasts. Biochem. Biophys. Res. Comm. 14: 109-112.
69. Nossal, N. G. and L. A. Heppel. 1966. The release of enzymes by osmotic shock from Escherichia coli in exponential phase. J. Biol. Chem. 241: 3055-3062.
70. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Ann. N. Y. Acad. Sci. 121: 321-349.
71. Pavlasova, E. and F. M. Harold. 1969. Energy coupling in the transport of B-galactosides by Escherichia coli. Effect of proton conductors. J. Bacteriol. 98: 198-204.
72. Rickenberg, H. W., G. N. Cohen, G. Buttin and J. Monod. 1965. La galactoside-permease d'Escherichia coli. Ann. Inst. Past. 91: 829-857.

73. Rickenberg, H. V., C. Yanofsky and D. M. Bonner. 1953. Enzymatic deadaption. *J. Bacteriol.* 66: 683-687.
74. Rosenthal, S., E. J. Reed and R. A. Weisman. 1969. Effect of lytic enzymes of Acanthamoeba castellanii on bacterial cell walls. *J. Bacteriol.* 98: 182-189.
75. Sapico, V., T. E. Hanson, R. W. Walter and R. L. Anderson. 1968. Metabolism of D-fructose in Aerobacter aerogenes: Analysis of mutants lacking D-fructose 6-phosphate kinase and D-fructose 1,6-diphosphatase. *J. Bacteriol.* 96: 51-54.
76. Schaeffler, S. 1967. Inducible system for the utilization of B-glucosides in Escherichia coli. I. Active transport and utilization of B-glucosides. *J. Bacteriol.* 93: 254-263.
77. Schaeffler, S. and W. K. Maas. 1967. Inducible system for the utilization of B-glucosides in Escherichia coli. II. Description of mutant types and genetic analysis. *J. Bacteriol.* 93: 264-272.
78. Schaeffler, S. and L. Mintzer. 1959. Acquisition of lactose-fermenting properties by Salmonellae. I. Interrelationship between the fermentation of cellobiose and lactose. *J. Bacteriol.* 78: 159-163.
79. Schaeffler, S., L. Mintzer and C. Schaeffler. 1960. Acquisition of lactose-fermenting properties of Salmonellae. II. Role of the medium. *J. Bacteriol.* 79: 203-212.
80. Shenin, R. and B. F. Crocker. 1961. The induced concurrent formation of  $\alpha$ -galactosidase and B-galactosidase in Escherichia coli B. *Can. J. Biochem. and Physiol.* 39: 63-72.
81. Simoni, R. D., M. Levinthal, F. D. Kundig, W. Kundig, B. Anderson, P. E. Hartman and S. Roseman. 1967. Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. *Proc. Nat. Acad. Sci.* 58: 1963-1970.

82. Simoni, R. D., M. D. Smith and S. Roseman. 1968. Resolution of a staphylococcal phosphotransferase system into four protein components and its relation to sugar transport. *Biochem. Biophys. Res. Commun.* 31: 804-811.
83. Sison, B. C., Jr., and W. J. Schubert. 1958. On the mechanism of enzyme action. LXVIII. The cellobiase component of the cellulolytic enzyme system of Poria vaillantii. *Arch. Biochem. Biophys.* 78: 563-572.
84. Sison, B. C., Jr., W. J. Schubert. and F. F. Nord. 1958. On the mechanism of enzyme action. LXV. A cellulolytic enzyme for the mold Poria vaillantii. *Arch. Biochem. Biophys.* 75: 260-272.
85. Sols, A. and G. de la Fuente. 1961. Transport and hydrolysis in the utilization of oligosaccharides by yeasts. p. 363-377. *In*: Kleinzeller, A. and A. Kotyk (ed.) *Membrane transport and metabolism. A symposium.* Academic Press, Inc., New York.
86. Tanaka, S. and E. C. C. Lin. 1967. Two classes of pleiotropic mutants of Aerobacter aerogenes lacking components of a phosphoenol-pyruvate-dependent phosphotransferase system. *Proc. Natl. Acad. Sci. U. S.* 57: 913-919.
87. Ulitzur, S. The transport of B-galactosides across the membrane of permeaseless Escherichia coli ML<sub>35</sub> cells after treatment with cetyltrimethylammonium bromide. *Biochim. Biophys. Acta* 211: 533-541.
88. Umezurike, G. M. 1969. Cellulolytic activities of Botryodiplodia theobromae *Pat. Ann. Bot.* 33: 451-462.
89. Umezurike, G. M. 1970. Cellulase and B-glucosidase components in culture filtrates from Botryodiplodia theobromae *Pat. J. Exp. Bot.* 21: 639-650.
90. Umezurike, G. M. 1971. The purification and properties of extracellular B-glucosidase from Botryodiplodia theobromae *Pat. Biochim. Biophys. Acta* 227: 419-428.



91. Veibel, S. 1950. B-glucosidase. p. 583-620.  
In: Sumner, J. B. and K. Myrback (ed.).  
The enzymes. Vol. I, part 1. Academic  
Press Inc., New York.
92. White, A., P. Handler and E. L. Smith. 1964.  
p. 46-47. Principles of Biochemistry. 3rd  
ed. McGraw-Hill Book Co., New York.
93. Wilson, R. W. and D. J. Niederpruem. 1967. Control  
of B-glucosidases in Schizophyllum commune.  
Can. J. Microbiol. 13: 1009-1020.
94. Winkler, H. H. and T. H. Wilson. 1966. The role of  
energy coupling in the transport of B-galac-  
tosides by Escherichia coli. J. Biol. Chem.  
241: 2200-2211.
95. Wood, T. M. 1968. Cellulolytic enzyme system of  
Trichoderma koningii. Biochem. J. 109::  
217-227.
96. Youatt, G. 1958. Fungal cellulases. IX. Growth  
of Stachybotrys atra on cellulose and  
production of a B-glucosidase hydrolyzing  
cellobiose. Aust. J. Biol. Sci. 11: 209-  
217.

## VITA

Ellen Oliver Smith was born June 3, 1945, in Nashville, Tennessee. She attended grade schools in Donaldson, Tennessee, Fort Worth, Texas, and Irving, Texas, and graduated from Irving High School in May, 1963. She received the degree of Bachelor of Science in Microbiology from Louisiana State University, Baton Rouge, Louisiana, in May, 1967. In September, 1967, she entered the Graduate School of Louisiana State University and is a candidate for the degree of Doctor of Philosophy in the Department of Microbiology.

Mrs. Smith is the wife of John Robert Smith.

## EXAMINATION AND THESIS REPORT

Candidate: Ellen Oliver Smith

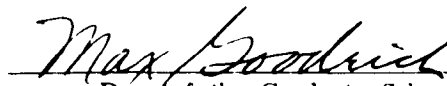
Major Field: Microbiology

Title of Thesis: Carbohydases of Alcaligenes faecalis (ATCC 21400)

Approved:

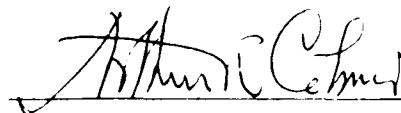
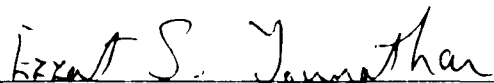
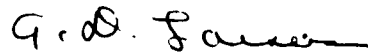
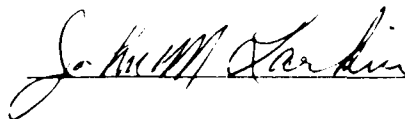


Major Professor and Chairman



Dean of the Graduate School

### EXAMINING COMMITTEE:



Date of Examination: April 4, 1972